CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

Vol. I. Steroid Hormones and Tumour Growth

and

Steroid Hormones and Enzymes

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CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

VOLUME I

Steroid Hormones and Tumour Growth
and
Steroid Hormones and Enzymes

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Assisted by
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With 48 Illustrations



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PREFACE

The Ciba Foundation is an international centre where workers active in medical and chemical research are encouraged to meet informally to exchange ideas and information. In the two and a half years since its opening in June, 1949, in addition to many part-day discussions, there have been 13 international symposia, each lasting two to four days, attended on invitation by outstanding workers from many countries.

The informality and intimacy of these meetings have permitted discussion of current and incomplete research and stimulated lively speculation and argument. They have also been the occasion for reference to much published and unpublished work throughout the world. The proceedings are now being issued in full, with only the minimum of editing, in order to pass on to a far wider audience the benefits of these meetings. Assembled in book form they present very readably much information not readily available elsewhere.

Nine of the first 13 Symposia form a series on "Colloquia on Endocrinology," dealing mainly with steroid hormone problems. One of these, on Nomenclature of Steroids, has had its conclusions published separately*; of the remaining eight, two are now combined in each of four volumes.

Volume I contains the proceedings of a colloquium on Steroid Hormones and Tumour Growth (Book I), and of another, even less formal, on Steroid Hormones and Enzymes (Book II). The former covers the induction of malignant growth by steroids, with special reference to the mammary gland, the use of steroids in cancer therapy and, in particular, the clinical and metabolic effects of ACTH and cortisone. Book II, on Steroid Hormones and Enzymes, is made up of

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summaries prepared by the programme speakers of the work on which their unscripted remarks at the colloquium were based. To these summaries the full general discussions have been added. The references provided in both sections of this volume should be of especial value to workers interested in these overlapping fields of research.

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Book II—STEROID HORMONES AND ENZYMES

Chairman : S. J. FOLLEY, DSc, PhD, FRS.

List of those participating in or attending the Conference on Steroid Hormones and Tumour Growth, 10th to 12th July, 1950

W. T. Astbury	University of Leeds
R. W. Begg	University of Western Ontario, Canada
R. R. Bomford	London Hospital, London
E. Boyland	Chester Beatty Research Institute, London
J. H. Burchenal	Sloan-Kettering Institute, New York
H. Burrows	*late of* Chester Beatty Res. Inst., London
A. Chamorro	Institut du Radium, Paris
G. W. Corner	Carnegie Institution of Washington, U.S.A.
A. T. Cowie	National Institute for Research in Dairying, Reading
L. Dmochowski	University of Leeds
K. Dobriner	Sloan-Kettering Institute, New York
S. J. Folley	National Institute for Research in Dairying, Reading
L. F. Foulds	Chester Beatty Research Institute, London
W. U. Gardner	Yale University School of Medicine
F. Gross	Ciba Limited, Basle
A. Haddow	Chester Beatty Research Institute, London
R. Hertz	National Institute of Health, Bethesda, U.S.A.
I. Hieger	Chester Beatty Research Institute, London
Lord Horder	London
E. S. Horning	Chester Beatty Research Institute, London
C. Huggins	University of Chicago
A. E. Kellie	Courtauld Institute of Biochemistry, London
R. Korteweg	Nederlandsch Kankerinstituut, Amsterdam
W. R. Lyons	University of California
O. Mühlbock	Nederlandsch Kankerinstituut, Amsterdam
B. D. Pullinger	The Glasgow Royal Cancer Hospital
C. W. Shoppee	University College of Swansea
I. F. Sommerville	University of Edinburgh
C. Chester Stock	Sloan-Kettering Institute, New York
P. C. Williams	Imperial Cancer Research Fund, London

List of those participating in or attending the Conference on Steroid Hormones and Enzymes, 8th to 10th March, 1950

I. E. Bush	National Institute for Medical Research, London
P. Mary Cotes	University of Cambridge
D. H. Curnow	Animal Health and Nutrition Laboratory, Western Australia
E. C. Dodds	Courtauld Institute of Biochemistry, London
L. A. Elson	Chester Beatty Research Institute, London
W. H. Fishman	Tufts College, Boston, Mass.
S. J. Folley	National Institute for Research in Dairying, Reading
T. H. French (decd.)	National Institute for Research in Dairying, Reading
Nancy Gough	Chester Beatty Research Institute, London
A. L. Greenbaum	University College, London
Lord Horder	London
C. D. Kochakian	Oklahoma Medical Research Unit and Hospital, Oklahoma City, U.S.A.
Patricia McLean	Courtauld Institute of Biochemistry, London
W. H. H. Merivale	Guy's Hospital Medical School, London
R. K. Meyer	University of Wisconsin, U.S.A.
G. T. Mills	University of Glasgow
J. N. Smith	St. Mary's Hospital, London
A. W. Spence	St. Bartholomew's Hospital, London
I. D. E. Storey	University of Edinburgh
R. H. S. Thompson	Guy's Hospital Medical School, London
A. Tickner	Guy's Hospital Medical School, London
H. G. Williams-Ashman	Chester Beatty Research Institute, London
F. G. Young	University of Cambridge

BOOK I

STEROID HORMONES AND TUMOUR GROWTH

FOREWORD

by

PROFESSOR A. HADDOW, D.SC., PH.D., M.D.

OF all the branches of physiology and chemistry having a direct bearing on the cancer problem, steroid endocrinology holds a key position, and is certain to figure prominently—possibly decisively—in the ultimate solution. It is not without significance, and is perhaps prophetic, that the first example of this physiological control of one type of human cancer, namely the œstrogen treatment of carcinoma of the prostate, should have sprung from this field. Even if the present situation is one of extreme complexity, with innumerable anomalies and paradoxes, the subject shows every sign of active growth, with each fresh development having an immediate impact upon one or other aspect of the tumour problem. In all these circumstances the Ciba Foundation conference on Steroid Hormones and Cancer served a valuable purpose. If recent years have seen advances, as for instance in our knowledge of the influence of steroid hormones on the appearance and behaviour of tumours of the mammary gland, pituitary and gonads, there is also need for constant revision, and it is refreshing to find, for example, that the moderately simple interpretation of an anti-androgenic mechanism in the control of cancer of the prostate, so far accepted, is itself now open to some question. The conference further provided useful discussion of the metabolic functions of the steroid hormones, of the tumour-host relationship, and of the nature and significance of alterations in the steroid excretion, leading especially to the newer view that the origin of neoplastic disease may be associated with an adrenal-gonad insufficiency of a hitherto unexpected kind. We owe a considerable debt of gratitude not only to

the Ciba Foundation itself and to those workers from the United States, Canada, the Continent and England who contributed authoritative papers, but also to Dr. G. E. W. Wolstenholme, Miss M. P. Cameron and Mr. J. M. Garratt for their work of editorship which has allowed the proceedings of the conference to be available in permanent form.

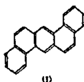
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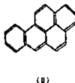
THE INDUCTION OF NORMAL AND MALIGNANT GROWTH WITH STEROIDS AND RELATED SUBSTANCES

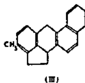
STEROIDS IN RELATION TO CANCER FROM THE CHEMICAL ASPECT

C. W. SHOPPEE

FOLLOWING the original production of malignant skin tumours in the laboratory with coal tar by Yamagiwa and Ichikawa in 1915, the work of Bloch, Passey, Leitch, Kennaway, Mayneord, Cook and Hieger led during the decade 1920-30 to the discovery of the carcinogenic hydrocarbons. 1:2:5:6-Dibenzanthracene (I), the first synthetic carcinogen, was soon succeeded in 1933 by 3:4-benzpyrene (II) and other polycyclic aromatic hydrocarbons. Because methyl-cholanthrene (III) can be regarded as a 5:6:10-trisubstituted-1:2-benzanthracene, Cook predicted that it would be carcinogenic, and Cook and Haslewood in 1934 showed it to be highly potent.


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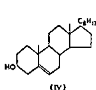

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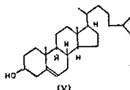
(III)

In 1932, the chemical structure of the steroids was elucidated and the formula of cholesterol (IV) settled (Wieland, Windaus, Rosenheim and King); this formula with the

addition of the stereochemical detail* shown in (V) is now completely established by the work of Wieland and Dane in 1933, Reichstein and Sorkin in 1946, and in 1947, by Shoppee and Cornforth and Robinson. Stereochemistry is immensely important in relation to biological activity.

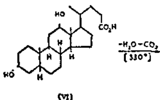


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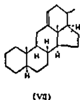
(V)

In 1933, Wieland and Dane converted deoxycholic acid (VI) via dehydronorcholene (VII) into methylcholanthrene (VIII). The high carcinogenic potency of methylcholanthrene and its close structural relationship to the steroids, which was fully appreciated by Cook even before the transformation (VI⟶VIII) was achieved, led to the suggestion associated with the names of Cook, Kennaway and Dodds that carcinogenic hydrocarbons might arise from steroids occurring naturally in the organism.



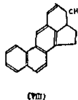
(VI)





(VII)





(VIII)

There is, however, no direct evidence that these reactions, although attainable *in the laboratory*, occur under physiological conditions. It was reported by Druckrey, Richter, and Vierthaler in 1941 that benzene extracts of *Bacillus coli* cultures grown in the presence of dehydronorcholene (VII) produced malignant tumours in rats; this activity is not due

*Full-line and broken-line bonds show the position of groups above or below

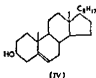
to some transformation product of dehydronorcholene because this substance, which is noncarcinogenic, is unaltered by *B. coli*, as shown by Butenandt and Dannenberg in 1950. It seems therefore that the transformation (VI⟶VIII) is not biologically significant, although (VI) may produce sarcomas in mice (Kennaway, 1940).

An alternative and smoother path for the conversion of a 17α-hydroxy-20-ketosteroid (IX), of the type produced by the adrenal cortex, *via* a D-homoandrostane derivative (X) to a carcinogenic methylchrysene (XI) was suggested by Shoppee in 1947; the stage (IX⟶X) proceeds under physiological conditions, but there is no evidence that this is also true for the complete dehydrogenation (X⟶XI), although analogies may be quoted, e.g. dehydrogenation of cyclohexanecarboxylic acid to benzoic acid in the liver (Bernhard, 1945; Dickens, 1948, 1949) and the *in vivo* dehydrogenation of (+)-œstrone to (+)-equilenin in the pregnant mare (Girard, 1932, 1933). The saturated hydrocarbon 17α-methyl-D-homoandrostane corresponding to (X) is not carcinogenic in mice by painting; the methylchrysene (XI) appears to be a weak carcinogen, for although it produced no tumours in mice by painting, by the graft technique of Horning it caused proliferation of the prostatic epithelium, accompanied by active mitosis in the glandular epithelium, with infiltration of the stroma, processes which in time might have led to tumour formation. Nevertheless, it would seem that the transformation (IX⟶XI) is not significant in regard to the development of "spontaneous" tumours.

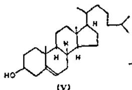
(IX) (X) (XI)

The analytical approach to the question of the production of carcinogenic hydrocarbons from naturally occurring

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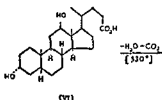


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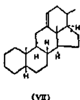


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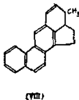
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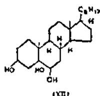
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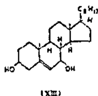
this time as to the mechanism of the connexion. The natural œstrogenic hormones induce normal growth of the uterine mucosa, but are all able to produce mammary cancer in genetically suitable male and female mice, and also pituitary, uterine, testicular, subcutaneous, leucocytic and bone tumours. The œstrogenic factor was isolated and identified by Doisy in 1936 as 3:17β-œstradiol, and the original observation of Lacassagne in 1932 for œstrone has been confirmed by Burrows in 1935, whilst experimental work leading to tumorigenesis has been reported for œstradiol (Gardner, 1936), and for equilenin and equilin (Lacassagne, 1936; Gardner, 1936). From the chemical point of view, it is not known whether œstrogens cause or mediate in cellular proliferation; their action is unspecific, inasmuch as many œstrogens of diverse and different chemical types are known, but all can produce tumours.

Reference must next be made to the striking experiments of Woolley, Fekete and Little (1940, 1945); in a strain (CE) of inbred mice, either ovariectomy of females or castration of males shortly after birth gave 100 per cent yields of adreno-cortical tumours which metastasized and were transplantable. Nothing extrinsic is administered to the mice and the tumour arises in a suitable genetic milieu by hormonal imbalance probably occasioned by adrenal hyperactivity in compensation for the absence of the gonads. Rhoads, Dobriner and their co-workers (1947) have suggested that adrenocortical hormone production and metabolism are very frequently disturbed in neoplastic disease. They have isolated from the ketonic fractions of human urines some 42 compounds, all steroids, of which at least 27 have been identified; one of these compounds, 3α-hydroxy-5-*iso*androst-9:11-ene-17-one (XV), appears to be associated with cancer. In Dr. Dobriner's words "a fair statement would seem to be that this compound is very significant but not wholly specific for cancer"; from the chemical point of view there can be no doubt that it is derived by dehydration of one of the 11β-hydroxy-steroids characteristic of the adrenal cortex, and which are subject to

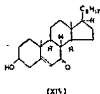
steroids is by examination of lipoid-soluble material from tumour tissue, or from unaffected organs or body fluids of cancer cases; in this way evidence of the occurrence of carcinogens in human tissues has been obtained by six independent groups of workers (Schabad, 1940; des Ligneris, 1940; Hieger, 1940; Sannié *et al.*, 1941; Steiner, 1942; Menke, 1942). The tumours produced by these tissue extracts are generally of the spindle-cell type resembling those produced by carcinogenic hydrocarbons, but no compound or compounds responsible have been isolated. In an investigation of a tissue extract from Bantu livers supplied by Dr. Hieger, the writer found more than 85 per cent of cholesterol, and a little of the *n*-paraffin $C_{29}H_{60}$, but the only other compounds as yet isolated and identified were oxidation products, probably harmless, of cholesterol, namely cholestane-3β:5a:6β-triol (XII), 7-hydroxycholesterol (XIII), and 7-ketocholesterol (XIV). It is interesting to note that the triol (XII) and the ketone (XIV) are produced when cholesterol is irradiated with X-rays in an aqueous medium, whilst the diol (XIII) results from auto-oxidation of cholesterol with molecular oxygen at 20°C. in colloidal aqueous solution. The main point in the present connexion is that Dr. Hieger and the author were unable to detect in the tissue extract and after removal of the ballast material, i.e. after about a hundredfold concentration, any substance giving the type of ultra violet fluorescence spectrum characteristic of polycyclic aromatic hydrocarbons.



(XII)



(XIII)



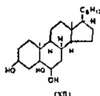
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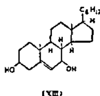
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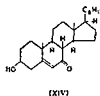
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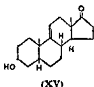
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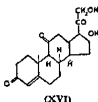
this time as to the mechanism of the connexion. The natural œstrogenic hormones induce normal growth of the uterine mucosa, but are all able to produce mammary cancer in genetically suitable male and female mice, and also pituitary, uterine, testicular, subcutaneous, leucocytic and bone tumours. The œstrogenic factor was isolated and identified by Doisy in 1936 as 3:17β-œstradiol, and the original observation of Lacassagne in 1932 for œstrone has been confirmed by Burrows in 1935, whilst experimental work leading to tumorigenesis has been reported for œstradiol (Gardner, 1936), and for equilenin and equilin (Lacassagne, 1936; Gardner, 1936). From the chemical point of view, it is not known whether œstrogens cause or mediate in cellular proliferation; their action is unspecific, inasmuch as many œstrogens of diverse and different chemical types are known, but all can produce tumours.

Reference must next be made to the striking experiments of Woolley, Fekete and Little (1940, 1945); in a strain (CE) of inbred mice, either ovariectomy of females or castration of males shortly after birth gave 100 per cent yields of adrenocortical tumours which metastasized and were transplantable. Nothing extrinsic is administered to the mice and the tumour arises in a suitable genetic milieu by hormonal imbalance probably occasioned by adrenal hyperactivity in compensation for the absence of the gonads. Rhoads, Dobriner and their co-workers (1947) have suggested that adrenocortical hormone production and metabolism are very frequently disturbed in neoplastic disease. They have isolated from the ketonic fractions of human urines some 42 compounds, all steroids, of which at least 27 have been identified; one of these compounds, 3α-hydroxy-5-*iso*androst-9:11-ene-17-one (XV), appears to be associated with cancer. In Dr. Dobriner's words "a fair statement would seem to be that this compound is very significant but not wholly specific for cancer"; from the chemical point of view there can be no doubt that it is derived by dehydration of one of the 11β-hydroxy-steroids characteristic of the adrenal cortex, and which are subject to

extremely ready loss of the 11β-hydroxyl group as a molecule of water (Shoppee, 1940). There is also the recent evidence (Hench, 1948) that Kendall's Compound E, or Reichstein's Substance F, *commonly known as cortisone (XVI), is highly* effective in the treatment of rheumatoid arthritis, and the suggestion has been made that steroid hormones may be *implicated quite generally in pathological conditions.* These matters are to be further discussed by Dr. Dobriner and Dr. Burchenal.



(XV)



(XVI)

Since anti-tumour activities of steroids are to be discussed by Dr. Stock, it will be sufficient to mention the suggestion of Lipschutz (1948) that steroid balance may be an auto-defensive anti-tumoural device, and to refer to the work of Professor Huggins on the effect of androgen withdrawal and the administration of œstrogen in prostatic carcinoma.

A brief reference to growth inhibition is desirable; it has been shown by Haddow (1937 *et seq.*) that there is some correspondence between the growth inhibiting property and the carcinogenic activity of various compounds. These observations relate to compounds of various chemical types, such as aromatic hydrocarbons, aminostilbenes, and both *aliphatic and aromatic nitrogen mustards, but not to steroids;* it is therefore of interest to recall the report of Heilman and Kendall (1944) on the effect of cortisone in inhibiting the growth of lymphosarcomata. There seems to be a considerable field of work here in the systematic examination of steroids for effects of this kind.

The work at the Royal Cancer Hospital on the inhibition of established tumours suggests that the primary effects of quite diverse chemical compounds may be fundamentally

similar and that inhibitory effects result from cross-linking of the protein fibres of the chromosomes, whilst the later appearance of tumours arises from the subsequent genetic change. In this connexion it is to be recollected that the 11-oxygen steroids of the adrenal cortex, e.g. cortisone, are considered to produce their biological effects by influencing protein metabolism (Long, 1942).

To a chemist, perhaps the most striking difference in the production of tumours by a so-called chemical carcinogen (e.g. hydrocarbons, heterocyclic aromatic compounds, azo-dyes, aminostilbenes, or nitrogen mustards) on the one hand, and by a virus (e.g. the Rous agent) on the other, is the time factor required; months are needed by the chemical carcinogens, but practically no time at all by the virus. The Rous agent appears from the available evidence to be a protein; that it is able to induce the change from normal cell to malignant cell at once suggests that this change involves protein synthesis.

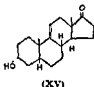
Haddow (1944, 1947) has suggested that a virus may possibly function as a substitute plasmagene: so that in an idealized model we have to envisage a bimolecular, cytochemical reaction between two proteins, the chromosome fibre and the substitute plasmagene, whereas if we introduce a carcinogen, e.g. methylcholanthrene, three chemical entities, chromosome, plasmagene, and carcinogen are concerned. The probability of a termolecular reaction is however about 1,000 times less than that of a bimolecular reaction, and to achieve the same reaction rate would require a very much lower termolecular activation energy.

A reaction rate k may be expressed by the relation

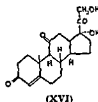
$$k \propto e^{\frac{-\Delta H}{RT}} \cdot e^{\frac{\Delta S}{R}}$$

where the $-\Delta H$ exponent relates to the heat of the reaction and the ΔS exponent relates to entropy. This latter is concerned with molecular configuration; if therefore a biological reaction requires molecular conformation of the

extremely ready loss of the 11β-hydroxyl group as a molecule of water (Shoppee, 1940). There is also the recent evidence (Hench, 1948) that Kendall's Compound E, or Reichstein's Substance F, commonly known as cortisone (XVI), is highly effective in the treatment of rheumatoid arthritis, and the suggestion has been made that steroid hormones may be implicated quite generally in pathological conditions. These matters are to be further discussed by Dr. Dobriner and Dr. Burchenal.



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DISCUSSION

[illegible]

produced in the organism. Dr. Boyland was the first to investigate the metabolism of this type of compound Dr. Boyland and later we ourselves were able to show that during the metabolism of the carcinogens, hydroxylated derivatives are formed which are not carcinogenic. The cholesterol metabolites Dr. Shoppee mentioned are also hydroxylated compounds.

[illegible]

hormones.

HUGGINS What bonds do you get between steroids and proteins?

SHOPPEE· That is a very difficult question. The most obvious answer is that the hydroxyl group at position 3 which occurs in all natural steroids and which is known, for example, to tend to form double molecules, seems to be implicated. As a matter of practical interest, it is extremely difficult to get a 3-hydroxyl steroid really free from [illegible]

reactants involving loss of molecular degrees of freedom, this will lead to a low rate of reaction. It seems thus that the viruses, regarded as plasmagenes, may fit some part or parts of a normal chromosome with varying degrees of difficulty, and in consequence produce their effects with varying speeds. Steroids appear especially interesting because they *seem to stand intermediately between a virus*, such as the Rous agent, and the chemical carcinogens in regard to the speed with which their biological effects are produced.

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of substances could be differentiated by the ratio shown between the minimal effective dose applied directly to the vagina and the minimal effective systemic dose. A substance which has not got the two hydroxyl groups might conceivably be a pro-œstrogen, and might be converted into a biologically active œstrogen after injection.

WILLIAMS: Most of the pro-œstrogens are relatively inactive compounds.

gr
that it may be a π-complex.

PULLINGER: I understand that two-thirds of the œstrogen in the blood is protein-bound. Do the chemists know what that protein binding consists of?

SHOPPEE I don't think they do.

HIEGER: Those who have a chemical background often express a wistful longing for the isolation of polycyclic aromatic compounds in the body as an explanation for human cancer. They need not be altogether depressed at the failure to isolate such compounds, because these might only have a temporary existence in the body, and then be rapidly converted to some hydroxy detoxication compound. It is probable that attention may soon be focused on quite a different type

SHOPPEE The dehydrogenations which have been accomplished in the laboratory are all *in vitro* reactions at high temperature. The one attempt at an *in vivo* reaction by Butenandt and Dannenbaum with dehydronorcholene gave completely negative results

SOMMERVILLE: With regard to molecular configuration, it is obviously important to determine whether there are in fact inherent structural requirements related to biological action, for example, the distance between hydroxyl groups or hydrogen-bond-forming groups in the œstrogens

SHOPPEE: I couldn't agree more that configuration is important. In regard to this theory of the critical distance of the two hydroxyl groups, personally I don't like it. It's far too simple and specific. And you can

Nature, 161, 809).

FOLLEY: We should bear in mind Emmen's belief that there are œstrogens and pro-œstrogens, that is, compounds inactive themselves which can be converted into œstrogens in the body. These two groups

THE RÔLE OF DIETARY TRACE FACTORS I HORMONE-INDUCED TISSUE GROWTH

R. HERTZ

THIS paper discusses certain studies on the necessity f particular dietary trace factors for the optimum tiss growth response to various steroid hormones, particularly t œstrogens. The general field of the dependence on nutrition factors for endogenous production of pituitary hormone a ovarian steroids will be omitted, because such dietary failur of endogenous hormone production in various nutrition deficiency states are highly non-specific effects. The di cussion will relate more to the effects of such dietary tra factors on the tissue growth response to administered ex genous œstrogens when given in dosages which should yie extreme or maximum tissue-growth responses. Thus, t hormone in these studies that we will describe is not a limiti factor; it is the capacity of the tissue to increase its ma which will be the limiting factor. There were two dieta factors concerned: biotin and folic acid.

Biotin is one of the more recently identified factors of t B-complex on whose physiological significance we have real very little information. We first came to know of the exi tence of biotin from the fact that when animals are fed a hig proportion of raw egg albumin in the diet they suffer a tox syndrome which has been termed the "egg-white injur response." Rats which have been subjected to such an eg white injury experiment are in extremely poor conditio with loss of hair and general debility; these rats die fro biotin deficiency. The biotin deficiency comes about i directly because the egg-white contains a specific prote factor which we term avidin. This avidin has the capacit to combine with the biotin in the diet to form an avidin-bioti

complex. This complex cannot be absorbed from the gastrointestinal tract and passes out in the stool. Thus, these animals become biotin-deficient.

We have found that avidin is present, not only in the albumin of the hen's egg, but in the egg of practically every species of bird that we tested. We tested over twenty of them, and found that the presence of avidin was not related to the taxonomic origin of the bird. Avidin was also found in the gelatinous material surrounding the frog's egg; this material is the biological homologue of the albumin of the hen's egg. We also found it in turtle's eggs. It seemed to us that here was a substance which warranted further study.

In association with Dr. Fraps and others we undertook an analysis of the avidin content of the genital tract of the actively laying hen. We have used as an assay method for avidin a microbiological test employing a yeast organism which requires biotin for growth. Avidin will inhibit that growth, and addition of biotin will restore the previously inhibited growth.

The avidin content of the albumin-secreting portion of the genital tract is extremely high, and when one goes just 1 mm. beyond the albumin-secreting portion into the shell gland area one no longer finds avidin. It is found, however, in the mesentery which supplies the albumin-secreting portion of the oviduct. We find this distribution of avidin only if the hen is actively laying. If the hen stops laying, as a result of seasonal change or old age, then avidin is no longer found in the genital tract. There is no avidin in a sexually immature animal. This suggested to us that we might try to induce avidin formation in the three-week old chick. I will omit the experimental details of these studies which are published elsewhere.

Fig. 1 represents the genital tract of an untreated three-week old New Hampshire Red chick. This is the size of the structure obtained when one administers stilbœstrol for a six day period at a maximum dosage of 1 mg. daily, or any other œstrogen in a comparable dosage equivalent. We have



Fig 1 Genital tract of chick before and after œstrogen administration.

To face page 131

increase in the biotin content. In other words, the presence of avidin in the genital tract does not seem to alter materially the blood level of biotin under these experimental conditions. The total biotin activity is shown here, namely the capacity of the serum to support the growth of various biotin-requiring micro-organisms such as *Lactobacillus casei*, *Saccharomyces cerevisiæ*, and several others which were used quantitatively to check our results.

The situation becomes complicated since Trager has described an additional factor which he refers to as F.S.F. a fat-soluble factor, which is present in the serum of many species of animals and which has the capacity to replace biotin for the growth of many biotin-requiring organisms. It is not chemically biotin (it is fat soluble rather than water-soluble) and we refer to it as the "fat-soluble factor" or as "pseudobiotin."

Now, when œstrogens are given to a bird a very substantial degree of lipæmia occurs. We have found, associated with that lipæmia, a substantial increase in the amount of this F.S.F. material. In the case of the genital tract (Table II), there is a rather interesting situation. If biotin is administered to a previously untreated bird there follows some increase in the biotin content of the genital tract. Also, with

Table II
Oviduct Biotin Levels in Chicks

Treatment	*No of Chicks*	*Oviduct*	
		Biotin	*F S F*
None	6	19 0	17·0
Stilbœstrol	8	25 0	38·0
Stilbœstrol, Progesterone	10	27 0	37 0
Biotin, Stilbœstrol	8	62 0	46·0
Biotin, Stilbœstrol, Progesterone	9	256 0	42·0
Biotin	5	57 8	37·4

used mainly stilbœstrol. The mucous membrane has become highly proliferated under œstrogen stimulation, and this is the origin of the avidin. Under stilbœstrol stimulation we get no avidin production whatsoever. However, if we add to the stilbœstrol 1 mg. of progesterone daily over the same experimental period, we find extremely high amounts of avidin in the albumin-secreting portion of the genital tract. This means that avidin production in the bird is brought about under conditions which are quite comparable to the luteal phase of the mammalian menstrual cycle, and that two hormones are involved in the induction of this specific secretory activity.

These observations made us feel that it would be worth studying what was happening in the blood under similar experimental conditions. In Table I there are some quantitative data concerning the biotin content of the serum of birds under various types of hormonal treatment. Again we used sexually immature birds, about three weeks of age. The biotin content of the untreated bird is 3·6 mμg. per ml. of plasma.

Table I

SERUM BIOTIN LEVELS IN CHICKS

Treatment	*Number of Chicks*	*Total Biotin Activity*	*Fat Soluble Factor*	*True Biotin Activity*
Untreated . .	15	3·6 (3·0–4·2)	3·8 (2·6–5·0)	1·3 (1·1–1·7)
Stilbœstrol . . .	12	22·0 (17–30)	15·0 (10–22)	8·8 (5·2–11·0)
Stilbœstrol and Progesterone .	8	24·0 (15·0–31)	11·0 (9·0–13·0)	10·0 (8·5–13·0)

Under stilbœstrol there is a very marked increase in biotin activity. If progesterone is added to stilbœstrol, and there is a reproduction of the endocrine condition in which the anti-biotin, avidin, is formed, we get no substantial further

increase in the biotin content. In other words, the presence *of avidin in the genital tract does not seem to alter materially* the blood level of biotin under these experimental conditions. The total biotin activity is shown here, namely the capacity of the serum to support the growth of various biotin-requiring micro-organisms such as *Lactobacillus casei*, *Saccharomyces cerevisiæ*, and several others which were used quantitatively to check our results.

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Biotin, Stilbœstrol	8	62 0	46 0
Biotin, Stilbœstrol, Progesterone	9	256 0	42·0
Biotin	5	57 8	37·4

stilbœstrol alone, or with stilbœstrol plus progesterone, i.e. the condition under which we get avidin formation, the presence of the avidin does not attract from the circulation any of the biotin content of the serum and there is no substantial increase in biotin activity. However, if with stilbœstrol exogenous biotin is given at the rate of 1 mg. per day (an excess sufficient to flood the organism) a little more biotin can be got into the genital tract. Moreover, when stilbœstrol and progesterone are given and avidin is formed in the genital tract, exogenous biotin is concentrated in the genital tract in enormous proportions. In other words, this specific glyco-protein substance produced under specific hormonal stimulation, can, under these conditions, attract a specific dietary factor into the rapidly proliferating and secretory tissue. Just what that means we are not able to say at this time, but from the standpoint of the physiology of tissue growth it is extremely interesting.

Our next observations were on the effect of folic acid deficiency on œstrogen response in various species. When the chick is deficient in folic acid there is only a slight growth response in the genital tract, the tissues being increased three or four times rather than forty or fifty times, as they would be in animals on a complete diet. We used animals deficient in riboflavin and pyridoxine as controls against such factors as reduced food intake and general debility, and found that such debilitating deficiencies interfered only very slightly with the tissue growth response to maximum doses of œstrogen. We found also that we could get any degree of quantitative response between a complete failure of response and a maximum response as we restored increasing amounts of folic acid to the diet, and that there was, therefore, a definite quantitative interrelationship between the level of folic acid intake in the bird and the tissue growth response to œstrogens. It is interesting, however, that in the folic acid deficient animal treated with œstrogen, there is lipæmia and hypercalcæmia, metabolic evidence of the utilization of the œstrogen, but the tissue growth seems to be suppressed in some way.

The monkey failed to show an œstrogen response when it was on a folic acid deficient diet. In the folic acid deficient animal there is complete pallor of the perineum, no turgidity, very little cornification of the vaginal epithelium, and, on microscopic section, no proliferative response in the genital tract despite ten days of treatment with very high dosage of œstrogen, which in the case of the monkey was œstradiol benzoate. Dr. Hisaw stated recently (private communication) that he has been able to carry a monkey along on

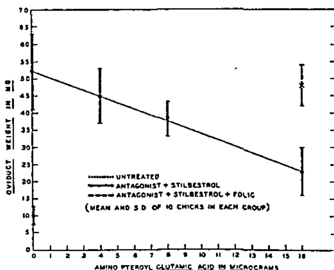


FIG. 2. Effect of aminopterin on œstrogen response in chick oviduct

œstrogen to get a proliferative endometrium; he then administered folic acid antagonists and precipitated menstruation by the interference of the folic acid antagonists with the utilization of œstrogens in the monkey. Dr. Goldsmith has shown that the folic acid antagonists could interfere with the frog oviduct response to œstrogens.

The chick oviduct is shown to be quantitatively inhibited in its œstrogen response in the presence of increasing amounts of folic acid antagonist (Fig. 2) A 48-hour chick oviduct

test was used which raised the weight of a day-old chick oviduct from 10 mg. to 50 mg. There is a quantitative interference with the response. The figure also shows the degree of restoration of that response when excessive amounts of folic acid are administered, even in the presence of this dosage of folic acid antagonist.

In the rat there is a little difficulty because a direct folic acid deficiency cannot be produced by dietary restriction because of the bacterial synthesis of folic acid in the intestinal tract. Therefore, folic acid antagonists have to be used. Eight of them have been used so far.

In the rat there is practically the same picture with respect to the uterine response, this time to œstradiol rather than to stilbœstrol. Ovariectomized sexually immature animals, whose uteri normally weigh 25–30 mg. each, average 80 mg. after 48 hours under œstrogen. Increasing dosage of folic acid antagonist brings this weight down almost to the level of the control. There is a slight residual tissue œdema which histologically appears to be simply an increase in interstitial fluid, thus giving a slight increase in weight. Histologically, the mitotic induction from the œstrogen is completely inhibited. Here also, when folic acid is administered in excessive dosage, provided the folic acid precedes the administration of the antagonist, we get a reversal of the inhibition. It is very essential that the folic acid be given before the administration of the antagonist, because if the antagonist, in this case aminopterin, be administered first it seems to become fixed in the tissue and cannot then be displaced. However, if the organism is previously flooded with excess folic acid the antagonist seems to have difficulty getting into its proper place. When the system is over-saturated with antagonist the restoration is poor, but just at the effective level there is practically complete restoration of response.

It should be emphasized that the tissue-growth responses to the steroid hormones are critically dependent and involved in a quantitative and specific way with dietary trace factors.

The important role of such a dietary trace factor as iodine has been appreciated for years and we know how critical such a minute trace factor is in thyroid function. We have come to appreciate more that these very potent dietary trace factors have a specific limiting role in the response to both endogenous and exogenous steroid substances. With the rapid development of the field of the antagonists to the B-complex factors we begin to have some tools for the control of hormone-induced tissue growth. From the standpoint of practical applicability we can say, from studies with breast cancer patients, that the available vitamin antagonists have such a narrow margin of safety that it is not feasible to try to employ this mechanism clinically. However, the biological principle is there, and perhaps with the development of more readily tolerable antagonists there may be some practical applications.

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DISCUSSION

BOYLAND: I worked for some years on the effect of sulphamethazine in causing hyperplasia of the testes of cockerels. A couple of years ago Dr. Hertz suggested that this might be due to the sulphamethazine having an anti-folic-acid-like action. We then tried to reverse this [illegible]

sulphamethazine.

purine antagonists would be worth study in this connection. Dr.

diaminopurine. This may indicate a mechanism by which the œstrogen induces the tissue growth reaction. We feel that by studying the effects of various antagonistic systems we may find additional information on the specific biochemical systems involved in œstrogen-induced tissue growth.

STOCK: Is that a true reversal with adenine, or is it blocking?

HERTZ: No

BURCHENAL: I think the citrovorum factor would be very interesting. It does seem to have more effect in preventing the anti-leukæmic effect of the antifolics. The folic acid always has to be given ahead of time, just as in your experiments, whereas with the citrovorum factor you can get considerable effect if it is given at the same time, or even an hour afterwards.

HERTZ: I understand that it is much more effective in general against the toxic effects of the folic antagonists

BURCHENAL: It seems to be. On the other hand, if you have got very

requires an entirely different set of nutritional factors. We have found that pyridoxine is critically required for androgen response in the seminal

specific responding end organ requires in higher amounts when responding to endogenous hormone.

FOLLEY: We have done some work on the response of the mammary gland to œstrogen in relation to aminopterin, and we have not been able to show any interference either with the action of endogenous

you use to obtain the results you showed with the rat?

HERTZ: An enormous dosage, 10 micrograms.

FOLLEY: And how much aminopterin would suppress the effect of that?

HERTZ: About 25 μg. would be sufficient to suppress completely the biological effect.

Your point about the general body growth is very pertinent. In studying the chick and the rat we used as controls animals with other

Cerecedo and others, does have deficient lactation, yet there is some

FOLLEY: Well, the mammary gland is certainly very sensitive to

per day and over that period the antagonist is so toxic that the rats die of anæmia.

What other folic acid antagonists have produced this inhibition of

HERTZ: I didn't have time to include the data on 2,6-diaminopurine.

on the specific biochemical systems involved in estrogen induced growth.

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the folic acid

Another point is that avidin is a potent antibacterial agent and almost all organisms require biotin for growth. You can take a frog's egg out of a very polluted pond and culture the internal part of the egg and find it sterile.

BURCHENAL: You mean that besides binding with biotin, keeping it from being absorbed through the gut wall, avidin also keeps bacteria from using the biotin?

HERTZ: Yes, and that is the basis of our microbiological test for avidin. It interferes quantitatively with the growth of any organism which requires biotin, and you get quantitative reversal with excess of biotin

BEGG: Is there any avidin in the mammalian organism?

HERTZ: The homologue of the albumin-secreting portion of the hen's oviduct is the middle portion of the Fallopian tube We tested mucosal scrapings from the Fallopian tube of the cow, the sheep, the rabbit, guinea pig, rat, and mouse, and we always found a little biotin, but never any avidin. This might, however, be due to contamination with blood, or to technical difficulties.

CORNER: Did you consider the time of the cycle?

HERTZ. In the rabbit, we tested in the post-copulatory period, and in the other mammals in the post-ovulatory phase of the cycle.

FOLLEY: Is there any avidin in the albumin around the rabbit ovum?

HERTZ. We have looked for avidin in the mucosa which secretes the envelope and have failed to find it.

HERTZ: In most of our studies we are dealing with a 2-4 day test period. *In comb growth studies we deal with an eight-day period* in the chick, and that is feasible.

BURCHENAL: Could you not scale your dose down?

FOLLEY: We've done that, and 6 μg. a day seems to be satisfactory for our rats as far as toxicity is concerned, but then you don't get any effect on the mammary growth.

HERTZ: There are very marked differences in tissues. Dr. Nelson at Berkeley showed me her studies on the effects of folic acid antagonists on the young of pregnant rats, beginning the administration of antagonist *early in the pregnancy. She has obtained extreme abnormalities* in fœtal development, fœtuses which are some three to four times

tissues.

WILLIAMS. Has anyone tried to apply those substances locally, intravaginally?

HERTZ: I don't know of any studies. It should be feasible.

WILLIAMS. Do chickens have corpora lutea, and do they produce progesterone?

HERTZ: Dr. Fraps at Beltsville has shown that a second steroid substance is required for ovulation in the bird, and when he gives p intravenously *in large dosage during the pre-ovulatory*

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the development of lymphoid tumours in mice yielded no uniform results.

The present experiments, which in part had originally been carried out in 1940 and 1941 and described by Dmochowski and Horning (1947), were undertaken to investigate the influence of œstrone alone and œstrone combined with castration on the lymphoid tissues of male mice of two partially inbred but non-homozygous, the so-called Hunt or "H" and MRC×Hunt or "R×H," stocks. The incidence of lymphoid tumours in female mice of these stocks is less than 1 per cent. The male mice of each stock were divided into three groups: those of the first were castrated and then painted with œstrone; those of the second were painted but not castrated; those of the third served as controls. Mice were castrated when three to four weeks old. Keto-hydroxy-œstrone in a 0·01 per cent solution in chloroform was applied twice weekly, starting one week after the operation, or at a similar age to the males which were painted only. Mice of the Hunt stock were painted for six months, and those of the "R×H" stock for four months.

The changes induced in the lymphoid tissues of the male mice of these two stocks were divided into three groups: those of the lymph nodes and other organs without changes in the thymus; those involving the thymus gland alone; those of the thymus, lymph nodes and other tissues and organs. The results are shown in Table I.

Table I

EFFECT OF ŒSTRONE AND ŒSTRONE PLUS CASTRATION ON LYMPHOID TISSUES OF MALE MICE OF "H" AND "R×H" STRAINS

Strain of Mice	*Experimental Procedure*	*Number of Mice Surviving*	*Mice with Lymphoid Changes Percentage*	*Mice with Thymus Changes Percentage*
"H"	Castration and Painting	65	58 5	18·4
	Painting	53	11 3	16 7
"R×H"	Castration and Painting	46	69 8	37·5
	Painting	48	18·8	33 3

THE INFLUENCE OF THE MALE AND FEMALE SEX HORMONES ON THE DEVELOPMENT OF LYMPHOID TUMOURS IN MICE

L. DMOCHOWSKI and E. S. HORNING

THE influence of the female sex hormone on the development of lymphoid tumours in mice was demonstrated by the observations of Mercier in 1938 and of Cole and Furth in 1941 that female mice of some strains have a higher incidence of lymphoid tumours than male mice of these strains. It was also demonstrated by the induction of these tumours in male mice with œstrogens (Lacassagne, 1937; Gardner, 1937), as well as by an increased incidence of lymphoid tumours as shown by Gardner, Kirschbaum and Strong in 1940; and by an earlier appearance of these tumours following œstrogen treatment (Shimkin, Grady and Andervont, 1941).

In connection with the significance attributed to the female sex hormone in the origin of lymphoid tumours in mice, several investigators carried out gonadectomy to ascertain its part in the development of these tumours. In some experiments such as those of Pybus and Miller in 1942, both ovariectomy and orchidectomy raised the incidence of lymphoid tumours. In other experiments, like those of Murphy (1944) and Law (1948) orchidectomy significantly altered the incidence, while ovariectomy had no effect. In yet another series of experiments, those of McEndy, Boon and Furth in 1942, orchidectomy failed to have an effect, and ovariectomy considerably lowered the incidence, while in the experiments of Gardner, Dougherty and Williams in 1944, and of Kirschbaum in 1944, gonadectomy had no significant influence on the appearance of lymphoid tumours in mice. This is by no means a full survey of the available literature, but it can be stated that the studies of the influence of gonadectomy on

and in the lymph nodes, as well as in other organs such as the spleen, liver, kidneys, or in the thymus alone. They consisted of enlargement of the thymus and/or of the lymph nodes with invasion of the surrounding tissues. Several tumours have been successfully transplanted, for many generations. There is no doubt about their truly malignant character. The first tumours were observed after six months, the latest after 15 months following the œstrone treatment. The difference between the present results and those of Gardner, Dougherty and Williams (1944) who observed no increase of tumours in their C_{57} black strain mice following œstrone treatment, may be due to sub-line differences. It should be mentioned here that Lacassagne considers œstrogens to be of greater

Table II

EFFECT OF ŒSTRONE PAINTING ON C_{57} BLACK MICE

	Control Mice			*Painted Mice*		
Sex	*Number of Mice*	*Number of Tumours*	*Incidence Percentage*	*Number of Mice*	*Number of Tumours*	*Incidence Percentage*
F	178	24	13.5	45	6	13.3
M	126	3	2.3	40	5	12.5

importance than heredity in the development of lymphoid tumours, while Gardner and his collaborators are of the opinion that the influence of sex hormones is conditioned by the genetic factor. We share this latter opinion. The genetic differences may have been responsible for the difference between the results of œstrone treatment of C_{57} black mice by Gardner and those of the present experiments.

Although no attempt was made in the present studies to assess the gradual changes in the lymphoid tissues under the influence of hormone treatment, there were indications that the amount of lymphoid tissue was decreased before the appearance of malignant changes, because in mice which died during the course of treatment, a decrease in the size of the lymph nodes and sometimes of the thymus and of the spleen

As can be seen from Table I, œstrone painting alone induces changes in the lymphoid tissues in the males of both stocks. The incidence of these changes was higher in the castrated and painted male mice. No changes were observed in the lymphoid tissues of 84 control mice. The changes in lymphoid tissues varied from hyperplasia to enlargement with invasion of the surrounding tissues. The earliest changes appeared after three months, the latest after twelve months from the beginning of the treatment.

Change in the thymus gland comprised enlargement of the gland, which consisted of enlarged cells with numerous mitoses, and was usually combined with invasive growth into the lungs, intercostal muscles, costal cartilages, pericardium, arteries, and into the trachea and diaphragm. All these changes were frequently accompanied by enlargement of the axillary, cervical, inguinal and mesenteric lymph nodes, combined with infiltration of the liver and kidneys, and with enlargement of the spleen. In some animals, however, the thymus was involuted. No changes were found in the blood of these animals.

It was not possible to establish whether these changes were of a truly malignant character because attempts to transplant the enlarged lymph nodes and the thymus gland were unsuccessful, possibly because the two stocks were not homozygous.

These experiments were followed up by another study of the influence of œstrone painting on males of a sub-line of C_{57} black low-cancer strain. They were painted, when four weeks old, with keto-hydroxy-œstrone in a 0·2 per cent solution in alcohol every other day for four months. The results are shown in Table II. As can be seen from the table, both male and female control mice develop lymphoid tissue tumours at an approximately equal average age of 12·5 months, varying from 5–22 months, although female mice show a higher incidence. The incidence of lymphoid tumours in male and female mice was approximately equal following œstrone treatment. The changes took place in the thymus

with similar invasive tendencies, and in a second rat we found a local malignant cystic tumour.

The incidence there is only about 2 in 100 in steroid-treated rats and none in control rats, so statistically it doesn't mean very much. The tumours occurred after two or three weeks of rather intensive therapy, so the time intervals are extremely small and it is highly unlikely that they are induced by testosterone. If anyone has any information on thymus tumours in rats I would be most pleased to have it. I wrote to Ingle and he could only give references to about five of them, and all of them had been associated with the administration of steroid hormones.

DMOCHOWSKI: It seems to me that too little attention has been paid to the genetic background of these changes. In the case of the mammary factor, we know now that there is a so-called inherited hormonal influence which is quite different from the hormonal factor which we have learned to know. Whether the same type of inherited hormonal influence may be responsible for the variable results of gonadectomy, and the fact that some mice give a higher incidence of lymphoid tumours than others I do not know. But I feel that geneticists may help the biologists and chemists a great deal.

HUGGINS: What was the incidence of these lymphatic changes in untreated animals?

DMOCHOWSKI: In the untreated II and R × II stocks, the incidence was below 1 per cent, so there was an amazing increase in these two stocks. The results may vary according to the kind of treatment; it is possible that the same amount of œstrogen supplied over a longer period may have a weaker effect of the thymus, for example over twelve months instead of three months. This is our observation and it agrees with that of Dr. Gardner and his colleagues.

HERTZ: In view of recent developments on the effects of steroids on connective tissue mesenchymal structures, might not the actual basis for the increased incidence be increased permeability of the ground substance by the lymphoid proliferating cells, rather than any direct effect on lymphoid tissue itself?

DMOCHOWSKI: Yes. It is very likely.

BEGG: In rats, while lymphoid elements themselves undergo dissolution with steroid hormone, the reticular material in the gland appears to undergo hyperplasia. It is difficult to follow because frequently you don't know whether you're seeing something which is hyperplastic and new or something which is uncovered because of disappearance of thymocytes. Phosphatase reaction gives a stronger reticular staining in the thymus after steroids than it does before.

DOBRINER: Can you stop œstrone application and still observe the same tissue changes?

DMOCHOWSKI: We did not stop the œstrone during the treatment, but the fact that quite a few of these tumours appeared within 12 months of the cessation of treatment indicates that once the process passes a certain threshold it becomes irreversible. Malignant changes

was noted. Furth (1946) suggested that the mode of action of œstrogens is to induce atrophy of lymphoid tissue, which is followed by regeneration. This suggestion and our own observations may have a bearing on the appearance of some tumours only 6–12 months after cessation of the treatment.

To conclude, it may be stated that the female hormone exerts a strong influence on the lymphoid tissues of male mice of a certain genetic background such as those of the strains examined, and the male hormone may have an inhibitory effect on the development of malignant changes of lymphoid tissues in these mice.

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DISCUSSION

STEROID HORMONES AND PROSTATIC CANCER IN MICE

E. S. HORNING

CANCERS of the breast, uterus and the prostate gland are of particular interest to the endocrinologist, not only because these are common sites of cancer in the human body, and are organs which are normally under endocrine control, but because the growth and behaviour of tumours derived from these particular organs can under certain conditions be influenced by means of hormonal therapy. Although, unfortunately, cancer of any of these organs cannot be permanently cured by this form of treatment, the most encouraging results have been demonstrated by Huggins and his school on the control of prostatic cancer in man by the application of anti-androgenic measures. It was not until the results of the investigations of Huggins and his co-workers (1941, 1945) were published that the influence of steroid hormones in the behaviour of prostatic cancer in man was fully appreciated.

In order to determine the effects of endocrine treatment on prostatic cancer in laboratory animals, it was first necessary to have at our disposal a transplantable glandular carcinoma of the prostate in pure line mice, similar histologically to those tumours of the prostatic gland which develop spontaneously in man. I propose to describe briefly the technique of subcutaneous homologous grafting by which these mouse prostatic tumours were obtained, and secondly to discuss the factors involved in obtaining successful grafts, before describing the influence of steroid hormones on the growth and behaviour of these transplanted tumours. I should like to make it clear at the outset that it is difficult to interpret these results obtained with mouse prostatic tumours in terms

DOBRINER: Have you any information on changes in size or morphology of the adrenals in your experiments?

DMOCHOWSKI: *We have no information about the behaviour of the adrenals. It would be a very interesting study because we do not know yet whether the action is direct, through the adrenals, or possibly even through the pituitary.*

DOBRINER: It was very interesting to me that in the beginning you got a decrease in lymphatic tissue, a decrease similar to that described with adrenal hormones. Dr. Woolley has found that adrenal size increases after œstrogen treatment. It seems to me that this may be a compensatory alteration in hormone production by the adrenal. Dr. Huggins, do you think that the œstrogen influence on tumours in humans is direct or indirect?

HUGGINS: It is direct

BEGG: In the rat thymus involution after administration of steroid hormones occurs in adrenalectomized animals. As far as the adrenal changes in these animals are concerned, you do get dissociation—after testosterone propionate you get tremendous losses in cholesterol, but practically none in ascorbic acid.

HERTZ: *Are œstrogens the only factors which will induce thymic atrophy in the adrenalectomized animal?*

BEGG: All the steroid hormones will produce involution in the adrenalectomized animal.

DOBRINER: Do they not produce overall decrease in the size of the tissues? Is this specific for the thymus?

thymic involution to a degree, and get change in adrenal size as well.

DMOCHOWSKI: *In connection with the involution of the thymus you mentioned, I can't understand why in some mice we get this marked enlargement of the thymus gland, and in other cases the thymus is just gone. Might it be an individual response through the adrenals in some of the mice?*

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DMOCHOWSKI. Yes.

prostatic epithelium are obtained from either the dorsal or ventral prostates of either strain A or strain C_3H mice. Under a dissecting microscope the selected pieces of epithelium are impregnated with crystals of the carcinogen, which in this instance was 20-methylcholanthrene, and the whole is implanted subcutaneously with the aid of a Bashford transplanting needle into host mice of the same age, sex and strain. Previous experiments have shown that since this technique involves homologous grafting, the reaction between the host and the graft plays an important role. In mice, the survival of an homologous graft seems to be dependent on the use of a closely inbred strain, as stock mice of indeterminate ancestry will not readily tolerate grafts of this kind. More recent experiments have indicated that the age of the donor providing the graft, and the age of the host mouse into which the grafts are implanted have an important bearing on the problem of graft survival. Another important factor is rapid vascularization of the graft in the subcutaneous tissues of the host animal.

A number of control grafts of prostatic epithelium without the carcinogen were implanted under the skin of host mice of the same age, sex and strain; these isolated fragments of prostatic epithelium continue to secrete prostatic fluid for as long as six months, provided that the grafts become successfully vascularized. The distension of the alveoli with fluid occurs to a degree far in excess of what is typical of the normal gland *in situ*, and invariably leads to a pronounced condition of cystic dilatation. In both the control and experimental grafts there is evidence that the secretion is slowly dispersed and absorbed within the tissues of the host. It will be noted that the prostatic epithelium in the control grafts is not hyperplastic, and hence it is important to distinguish as clearly as possible between growth in the grafts containing the carcinogen—this growth being due primarily to a failure in the release of the secretion, since ducts are no longer present—and proliferation which might be due to the direct action of the carcinogen.

of human prostatic cancer—but nevertheless they are of interest when studying the origin and cause of cancer in general. These mice experiments have, as we shall see, brought to light some facts of interest, amongst which is that the differences in response to endocrine therapy can be correlated with the histological structure of the particular tumour.

The technique of rapidly inducing tumours from adult prostatic tissues (impregnated with a carcinogen prior to subcutaneous implantation into host mice) was described by Horning in 1946. Since then Pan and Gardner (1948) of Yale have succeeded, by using the same methods, in inducing adenocarcinomas from adult mouse uterine epithelia impregnated with 20-methylcholanthrene. Also Boland Hughes (1949) of Pennsylvania University has obtained transplantable carcinomas of the bladder and adrenal cortex from homologous grafts combined with a carcinogen.

The mouse prostate gland, like that of the human, arises as a series of outgrowths from the embryonic urethra near its point of origin from the urinary bladder. The mouse gland consists of paired anterior, ventral and dorsal lobes, together with a small median gland, which may be absent or considerably modified in certain strains of pure line mice. Unlike the human gland, the mouse prostate has no uterus masculinus, nor is it encapsulated. A difference also exists in the number of ducts which open independently into the urethra. Thus in the human there are as many as 32, whilst in the mouse there are only six The prostatic epithelium is almost identical in its histology with that of the human. Hyperplasia of the prostate gland, however, never occurs spontaneously in rodents, as in man, but a similar condition sometimes arises in mice following prolonged treatment with œstrogens, which induces a pronounced metaplasia of the prostatic epithelium in the dorsal and ventral lobes, involving as in man urethral obstruction, retention of urine and hydronephrosis.

The technique of subcutaneous homologous grafting of prostatic epithelium is relatively simple. Small strips of

prostatic epithelium are obtained from either the dorsal or ventral prostates of either strain A or strain C_3H mice. Under a dissecting microscope the selected pieces of epithelium are impregnated with crystals of the carcinogen, which in this instance was 20-methylcholanthrene, and the whole is implanted subcutaneously with the aid of a Bashford transplanting needle into host mice of the same age, sex and strain. Previous experiments have shown that since this technique involves homologous grafting, the reaction between the host and the graft plays an important role. In mice, the survival of an homologous graft seems to be dependent on the use of a closely inbred strain, as stock mice of indeterminate ancestry will not readily tolerate grafts of this kind. More recent experiments have indicated that the age of the donor providing the graft, and the age of the host mouse into which the grafts are implanted have an important bearing on the problem of graft survival. Another important factor is rapid vascularization of the graft in the subcutaneous tissues of the host animal.

A number of control grafts of prostatic epithelium without the carcinogen were implanted under the skin of host mice of the same age, sex and strain; these isolated fragments of prostatic epithelium continue to secrete prostatic fluid for as long as six months, provided that the grafts become successfully vascularized. The distension of the alveoli with fluid occurs to a degree far in excess of what is typical of the normal gland *in situ*, and invariably leads to a pronounced condition of cystic dilatation. In both the control and experimental grafts there is evidence that the secretion is slowly dispersed and absorbed within the tissues of the host. It will be noted that the prostatic epithelium in the control grafts is not hyperplastic, and hence it is important to distinguish as clearly as possible between growth in the grafts containing the carcinogen—this growth being due primarily to a failure in the release of the secretion, since ducts are no longer present—and proliferation which might be due to the direct action of the carcinogen.

The neoplastic changes which occur in grafts implanted with the carcinogen will now be briefly described. Owing to the fact that the carcinogen is placed in direct contact with the living tissue, there is very little foreign body reaction or necrosis within the graft. Thus it is possible to study serial sections of the primary grafts, and to trace each invading clump of malignant cells back to the individual hyperplastic alveolus from which it has arisen.

Examination of prostatic grafts of various ages after implantation has shown conclusively that neoplasms are derived only from the alveolar epithelium which has entered the exhaustion phase of the secretory cycle. It is in these alveoli that hyperplastic epithelial changes are first seen, following a phase of mitosis, abnormal cell division and pycnosis. The hyperplasia is prominent in grafts four to five weeks old, giving an appearance to the epithelium which is closely similar to that seen in the human prostate during benign enlargement of the gland. In no single instance in any of the numerous grafts examined has the actively secreting epithelium lining the distended alveoli been the focus of malignant change. It is therefore presumed that the non-secreting exhausted alveolar cells are more susceptible to the action of the carcinogen. The hyperplastic changes in the mouse prostate are invariably accompanied by a pronounced increase in the fibromuscular stroma, and in some implants there is a lymphocytic infiltration which varies considerably in different grafts of the same age. In grafts six to eight weeks after implantation, the alveoli contain patches of epithelium which are 10–25 cells in depth, with cellular proliferation predominant in the basal layers.

At this early stage it is possible to distinguish three distinct types of epithelial proliferation. By studying the cytology and mode of growth of these early invasive cells through later phases of development in older grafts, it was possible to predict the type of tumours which would have arisen subsequently. The tongue-like colony of early malignant cells of the Type A is typical of those tumours which finally developed into

secreting glandular carcinomas. The Type B proliferation leads to the formation of a squamous-cell carcinoma in which alveolar formation is preserved. The more uncommon variety is the Type C lesion which arises from the duct epithelium and is characterized by a stratified squamous metaplasia *in situ*. In some grafts two varieties, types A and C of malignant change, co-exist in a primary graft and subsequently the tumour becomes a squamous-cell carcinoma, which rapidly infiltrates the adenocarcinomatous areas until all trace of the glandular tumour is lost. Both the glandular and the squamous-cell carcinomas grew on transplantation, and grew as serial transplants for many generations.

The observation that the focus of malignant change is restricted to the epithelium of the alveoli in the exhaustion phase of the secretory cycle is of importance. It would appear that the non-secreting alveolar epithelium is more susceptible to the action of the carcinogen than when at the height of secretory activity. These findings are also of interest because they support Haddow's (1947) contention that chemical carcinogens act more readily on cells following depression of cellular activity.

In view of the fact that œstrogens rapidly inhibit the secretion of the prostatic epithelium, and induce squamous metaplasia of the rodent prostate gland *in situ*, a series of experiments was undertaken to determine the influence of stilbœstrol alone, and stilbœstrol combined with the carcinogen in prostatic grafts growing in strain C_3H and strain A mice. In order to compare the depressant action of the œstrogen with the contrary action of the male sex hormone, which stimulates secretion of the prostatic epithelium, a number of grafts were prepared with the carcinogen alone and also with the carcinogen in combination with stilbœstrol and testosterone propionate. The results of these experiments are set out in the table on p. 36.

It was of interest to note that eight out of the 13 prostatic tumours induced with the carcinogen alone were glandular carcinomas. The remainder were all squamous cell tumours.

On the other hand, 23 of the tumours induced with stilbœstrol combined with the carcinogen were *squamous cell carcinomas*, and the remaining three were spindle cell sarcomas. As the sarcomas were far advanced it was impossible to ascertain by histological examination whether the tumours arose from the stroma of the graft or from the connective tissues of the host-bearing animal. Only three tumours, none of which were glandular carcinomas, were induced with the male sex hormone combined with 20-methylcholanthrene. Two were squamous cell growths, the remaining lesion being a spindle-cell sarcoma. These results are of significance inasmuch as they show that the inhibiting action of stilbœstrol on the

Group	Substances included with the graft	Strain	Total No bearing grafts	No. which developed tumours
I	20-Methylcholanthrene	A and C_3H	35	13
II	Methylcholanthrene and Stilbœstrol	A and C_3H	[illegible]	26
III	Methylcholanthrene and Testosterone propionate	A and C_3H	35	3

secretory epithelial cells appears to render them more susceptible to the action of the carcinogen. Assuming this to be the case, it would help to explain why the focus of malignant change is always restricted to the non-secreting epithelium.

The glandular carcinomas induced from prostatic grafts by 20-methylcholanthrene alone are similar in many respects to the uterine adenocarcinomas induced by Pan and Gardner (1948), who used a similar carcinogen and employed the same technique of tumour induction, except, however, that these prostatic tumours secrete an enormous amount of fluid. Histologically the primary glandular carcinomas consist of well differentiated alveoli lined by a low columnar epithelium actively engaged in secretion. Unfortunately these glandular carcinomas are apt to undergo squamous differentiation during serial transplantation. A few of these tumours underwent this change during their third passage, whilst into

exhibited no evidence of squamous differentiation until their tenth generation of serial transplants.

The influence of orchidectomy was determined on the growth and behaviour of these glandular carcinomas as well as on those glandular tumours which had undergone squamous differentiaton during serial transplantation. In every instance both types of tumours were transplanted into male mice of the same strain from which the primary grafts were derived. Every host mouse receiving these transplants was castrated before puberty, and was approximately four to six months of age at the time of transplantation. The following silhouette chart (Fig. 1) illustrates the influence of bilateral orchidectomy on the glandular carcinomas.

The control groups are shown on the left hand side, which shows the same tumour transplanted into intact normal mice, and it was clearly seen that the tumours grew rapidly during the first five weeks after transplantation, so rapidly in fact that several of the host bearing mice had to be killed owing to the size of the tumours. On the right the effect of bilateral orchidectomy is seen on this tumour. All the tumours regressed with the exception of two which grew at approximately the same rate as those in the controls. Testosterone propionate was administered to three host mice bearing rapidly regressing tumours, and in one instance the tumour resumed its normal rate of growth. Further experiments have shown that the influence of orchidectomy on the growth of these glandular carcinomas is extremely variable, and so is their response to treatment with the male sex hormone.

The next silhouette chart (Fig. 2) illustrates the effects of castration on the squamous cell growths, and it is of interest to note that in no single instance did any of these tumours show any marked inhibition of growth when compared with those growing in the intact mice of the control group. This demonstrates that withdrawal of androgens does [illegible] appear to influence prostatic tumours once they have [illegible]gone squamous differentiation. It is therefore possible carcino[illegible]

TRANSPLANTABLE GLANDULAR CELL CARCINOMA OF THE PROSTATE GROWING IN STRONG A MICE.

CONTROLS

No. 14/3 21/3 28/3 3/4 10/4 17/4 24/4 29/4

1 ♂
2 ♂
3 ♂ KILLED 12/4
4 ♂
5 ♂
6 ♂ KILLED 12/4
7 ♂
8 ♂
9 ♂
10 ♂ KILLED 12/4

CASTRATED

No. 14/3 21/3 28/3 3/4 10/4 17/4 24/4 29/4

1 ♂
2 ♂
3 ♂
4 ♂ TESTOSTERONE PROPIONATE
5 ♂ TESTOSTERONE PROPIONATE
6 ♂
7 ♂
8 ♂
9 ♂
10 ♂ TESTOSTERONE PROPIONATE

FIG. 1.

to correlate the histological structure of these prostatic tumours with their response to androgens.

It is difficult to say why a secreting prostatic adenocarcinoma, once it has undergone squamous differentiation,

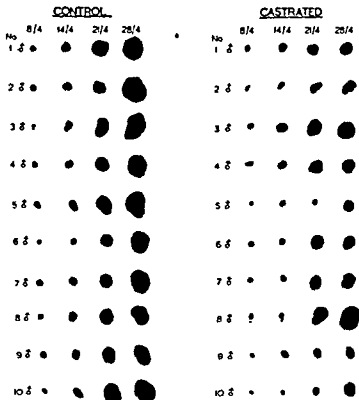


FIG. 2.

should cease to be dependent for sustained growth on an adequate androgen level. It is still more difficult to understand why these secreting adenocarcinomas should suddenly undergo differentiation into squamous-cell growths. Pullinger

(1949) in a recent paper on "Functional Differentiation in Mammary Tumours" draws attention to the squamous differentiation in these prostatic tumouts, and compares it to a similar phenomenon which occurs in the mammary glands of mice under experimental conditions. She contends that these changes might be due to an *intracellular œstrogen-like steroid.*

Summary

(1) The factors involved in obtaining successful subcutaneous homologous prostatic grafts are: (*a*) The use of a closely inbred strain of mice in which the grafts are more readily tolerated than they are in mice of indeterminate ancestry; (*b*) The rapid vascularization of the donor graft in the tissues of the host bearing animal; and (*c*) The age of the donor mouse providing the graft and the age of the host mouse into which the grafts are implanted; these factors have an important bearing on graft survival.

(2) Chemical carcinogens act more readily on cells following *depression of cellular activity. In grafts treated with the* carcinogen alone, the focus of malignant change has in every *single instance been restricted to the epithelium of alveoli* which have entered into the phase of secretory exhaustion.

(3) Tumours arise more rapidly and in greater numbers in prostatic grafts treated with the female sex hormone plus the carcinogen than they do in similar grafts which have been impregnated with the male sex hormone combined with the carcinogen, or in grafts treated with the carcinogen alone. It appears that stilbœstrol, by inhibiting secretion of the prostatic epithelial cells, renders them susceptible to the action of the carcinogen 20-methylcholanthrene.

(4) A number of secreting adenocarcinomas of the prostate, induced from homologous subcutaneous grafts of prostatic epithelium, regress when transplanted into mice castrated at puberty, and a smaller percentage of these tumours resume growth when the host mouse is treated with testosterone propionate. Tumours which at the time of their induction were diagnosed as squamous growths, or glandular carcinomas

which had undergone spontaneous squamous differentiation during serial transplanation, exhibit no appreciable response to this form of therapy. These results indicate that only glandular-cell carcinomas, and not squamous carcinomas, are dependent for their sustained growth on an adequate androgen level. This further suggests that the differences in response of the prostatic carcinomas in mice to hormonal therapy can be correlated with the histological structure of the particular tumour.

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DISCUSSION

HUGGINS: What little we know of cancer of the prostate has been learned almost entirely from human beings, there having been no

with no carcinoma, and others in the "exhaustion phase," developing carcinoma. I would place a little different interpretation on that. I have come to believe that the presence of tall cylindrical prostatic epithelium in an organism signifies that androgen is present in an

epithelium. I think that that is functionally better than this thin

The picture is complicated by the fact that carcinogen plus stil-

of these types. Stra

has been treated by

glycolysis in large m... the tumour. The normal columnar epithelium has no aerobic glycolysis. So I tend to think that if œstrogen is in excess the cell is functionally quite different from when androgen is in excess.

I ... thinks that secretion develops

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...ally active epithelium.

... of early tumour formation

carcinogen

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grafts and to trace each invading group of malignant cells back to the individual hyperplastic alveolus from which it had arisen; in every ... epithelium was the focus of malig-

All I can say is that these

ibiting the secretion of the

e susceptible to the action

of the carcinogen; I can offer no other interpretation. It should also be borne in mind that many workers contend that chemical carcinogens act more readily on cells following depression of cellular activity, and that the depressant action of stilbœstrol in the prostatic epithelium in the mouse prostate has been the subject of an extensive cytological study.

Secondly, Dr. Huggins asks whether I think the alveolar epithelium in the exhaustive phase of the secretory cycle in the prostate gland is physiologically active. My answer is, yes. The same phenomenon occurs during the secretory cycle in the mammary gland. In both the prostate and mammary glands of rodents the exhaustion phase of secretion is followed by a recovery phase, in which the glandular epithelium gradually enters into the secretory cycle again. As far as I am aware, little is known concerning the factors controlling the cyclic rhythm of secretion in either the prostate or the mammary glands.

ASTBURY: What is the significance of this aerobic glycolysis found in œstrogen-treated prostate?

HUGGINS: In general, cells with strong aerobic glycolysis are closer to the malignant, although not actually malignant, than cells without aerobic glycolysis.

BEGG: But there are really normal cells which do have high aerobic glycolysis.

HUGGINS: It can be debated whether that is not due to artifact.

glycolysis.

Begg: What about papilloma and normal skin, Dr. Boyland? I believe

Aerobic glycolysis is not specific, but it may be an indication.

Begg: But if, for instance, you give testosterone to a rat you will find a change in the alkaline phosphatase activity of the kidney. There is a very definite metabolic change in the kidney as a result of giving the steroid hormone. I don't think anybody infers from that that the tissue change has been towards malignancy. I realize that this is a very debatable point, but I just wondered at Dr. Huggins using this as a criterion.

Huggins: I think Dr. Boyland has expressed my opinion much more

origin.

Haddow: Are your metabolic results consistent with Boyland's?

where you have both potentialities in the cells, for secretion and for

who had escaped from anti-androgenic treatment with œstrogens. I would like to ask Dr. Huggins and the others here whether they have had any experience with progesterone.

Huggins: We did try progesterone in 1940, perhaps in insufficient amounts, but we observed no effect

Hertz: Dr. Trunell used 200–250 mg. per day.

Haddow: This might suggest that progesterone was effective only after œstrogens.

Hertz: No, not that it is effective *only* after œstrogens But the patients he felt justified in studying were only those who had escaped from the beneficial effect of œstrogen and orchidectomy.

Burchenal: We have been interested in the problems of the resistance of tumours to various chemotherapeutic agents, and I was very

castration. Was there any morphological difference in those tumours from the ones that did respond well, and did you try transplanting those resistant tumours to other mice?

Begg: It has been suggested that prostatic cancer patients who, after several years of favourable treatment, suddenly become refractory to treatment, may be getting androgens from some other source. Is it possible that even in the mammal there is a cellular adaptation to its environment, just as we know is found in bacteria and certain other organisms, which will change their enzyme systems, and their requirements for certain substances, depending on how long they have been

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orchidectomy were glandular carcinomas which had undergone squamous differentiation during transplantation. It was interesting also to observe that the glandular tumours which atrophied in response

... to consist of

None of these

Begg: Have you done any enzyme studies on these adenocarcinomas that have undergone squamous metaplasia? Acid or alkaline phosphatase?

Horning No, so far I have not done so.

Williams I wasn't *quite clear about the malignant change occurring* in the exhaustion phase. Was the existence of secretory exhaustion deduced from cytological evidence, or just from the fact that there wasn't any secretion in the alveolus? Because, in the end, malignant change would prevent secretion.

Horning: Whether or not any particular alveolus has entered into the exhaustive phase of the secretory cycle, is determined by the histological appearance of the glandular epithelium. The cytological

aspect of this problem has been studied extensively by many investigators.

HUGGINS: I want to point out that squamous carcinoma is one of the [illegible] begun to grow?

HORNING: No, I transplanted these tumours into mice which had been castrated before puberty.

FOULDS: There is a considerable resemblance to some mammary tumours which I am down to talk about tomorrow. Some of those mammary tumours were transplantable only into female mice or into [illegible] independent of the œstrogen, sometimes after one or two passages, [illegible]

Are these mammary tumours very slow growing?

FOULDS: Fairly.

HORNING: Prostatic tumours grow very rapidly. In some instances the glandular tumours following squamous differentiation, after they cease to become dependent upon androgens for sustained growth, grow extremely rapidly.

BEGG: In castrated animals are the grafts less vascular than in normal animals?

HORNING: These animals were not castrated.

CARCINOGENIC ACTIVITY OF STEROLS

I. HIEGER

IN 1947 we published a paper the title of which was "Carcinogenic activity of preparation rich in cholesterol" and in 1949 a paper entitled "Carcinogenic activity of lipoid substances." Since only a few new facts are available at the moment, my remarks will therefore be more in the nature of a very b.. f résumé of the present state of the investigation. It will hardly be necessary to say more than a word or two on the history of the subject. We would say that the development of syntheti cancer-producing compounds led to the successful search or naturally occurring carcinogens by Russian workers in 1937, when Schabad discovered the carcinogens present in The fa he livers of human subjects who had died of cancer. The fa he livers of human subjects who had years by investigators i's were followed up in the next few sources of carcinogen wer ther countries, who showed that liver, in human tissue othe be found also in non-cancerous rich (85 per cent) fractions o han liver, and in the cholesterol cholesterol was found to be ich tissues; finally, commercial producing agent. Just before no means inefficient cancer Shoppee, who had been with last stage of the research, suaded by Sir Ernest Kennawa chstein in Basel, was per forces working on the problem. come over to join our Beatty Institute, where he prepar come to the Chester an elaborate series of processes. T ee came to the Chester gens of the 300 or so synthetic ery pure cholesterol by produce cancer in 100 per cent of more powerful carcino- Commercial cholesterol and the chunds available will human tissues will induce 4–7 per ce. a very few months. months. However, if the effective nu ol rich fraction of ancer in mice in 1 of mice is used for

percentage calculation, that is, the incidence of cancer (sarcoma by subcutaneous injection) in the mice which survive the average latent period, the incidence then rises to 20 per cent. Shoppee's purest cholesterol produced one solitary sarcoma in 25 mice. This result very well illustrates the main difficulty of the work; the incidence is low, hence unless we make the quite gratuitous assumption that the mice are identically susceptible (and I fear that many investigators in cancer research *do* make this innocent assumption), work with large numbers of experimental animals is necessary. The interesting thing is that the non-cholesterol fraction from Shoppee's preparations, where the impurities were concentrated about ten times, did not produce a single tumour in 30 mice.

Furthermore, simple acetone crystallization of commercial cholesterol produced fractions, of which the least acetone soluble fraction gave tumours in four mice from a series of 20, while the untreated cholesterol gave a yield of two tumours in 30 mice.

In all, 27 sarcomas have been obtained from 450 mice injected with cholesterol not specially purified (the commercial product is labelled "re-crystallized"). In conversation with fellow workers the remark has frequently been made to me, that the impurities are responsible for the carcinogenicity of cholesterol. This assumption has one serious weakness; there is no evidence for it.

At any time, new data might alter one's perspective in this long term investigation, but at the present moment, if we might be so rash as to venture a prophecy, I would be inclined to say that fractionation of cholesterol will not yield any carcinogen with a potency as spectacular as the synthetic hydrocarbons; secondly, that individual susceptibility of the test animals is of the first importance; and thirdly, and this is the wildest guesswork, that the lipoidal focus attracts and kills mobile cells which liberate their fats as acids, and thus sets up a permanent subacute inflammatory region leading to neoplasia.

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hydrochloric acid in the body, but it doesn't burn holes in the body. We have to consider its dilution, the permanency of its location, and no doubt many other circumstances.

HADDOW: I take it that normally we probably have a considerable degree of interchange and conversion going on all the time, whereas

and progesterone are derived from cholesterol, but the site of the transformation (? the adrenal cortex) is not certain. I think it's

act that a low cholesterol, for where the sterol content must be very low

naphthylamine. We injected these substances in the same sample of olive oil, obtained from one source. We didn't get any tumours with the β-naphthylamine but we got about 50 per cent of tumours with the α-naphthylamine. In control experiments, with the same olive oil we

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DISCUSSION

… to what … the … are … here … ited … nge, but as to the identity of that substance, there is no information at all.

GARDNER: How are the cholesterol, or the cholesterol derivatives, administered?

HIEGER: A saturated solution in lard is injected subcutaneously. A … added when … naintain a

… llets? We … e dilution experiments, and so far I have never seen a tumour around a cholesterol or a stilbœstrol-cholesterol pellet, although when injected in sesame oil, we have seen tumours.

HIEGER: It is quite possible that the solvent plays a part in the formation of tumours. Lard itself gave no tumours when 350 control animals were injected. We were congratulating ourselves on this … experiment when an animal in the next batch of 50 suddenly … 400

… 1 in 400.

KELLIE: Is the so-called "commercial" cholesterol of animal origin?

HIEGER: Yes, from the spinal cord.

KELLIE: It has always impressed me that in a normal healthy person you get some 200–300 mg. per cent of cholesterol circulating through … stream and that most of the cells of the body must be in … In con- … per cent, … mour for- … seems to … irculating … when it is … but the … s a lot of

hydrochloric acid in the body, but it doesn't burn holes in the body. We have to consider its dilution, the permanency of its location, and no doubt many other circumstances.

DOBRINER: How much did you give?

HIEGER: A total of about one-third of a gram was given. They were injected approximately every three weeks—just as often as was necessary to maintain the nodules under the skin.

HADDOW: I take it that normally we probably have a considerable degree of interchange and conversion going on all the time, whereas here we have a depot, a good fraction of which remains there without effective interchange. While Shoppee and Dobriner very much doubt the possibility of conversion of a naturally occurring steroid *in vivo* to an aromatic polycyclic hydrocarbon, nevertheless Cook still believes that the process is feasible. Is it not possible, Dr. Shoppee, that a conversion of that kind might be greatly facilitated in a local, highly artificial concentration of cholesterol to which, as Hieger said himself, is added such a factor as inflammation? Is it not possible that a sufficiently small degree of conversion, yielding possibly only a few gamma of a particular aromatic substance, might explain the result?

SHOPPEE: I think the only available evidence, from tracer work, is [illegible]

transformation (? the adrenal cortex) is not certain. I think it's extremely difficult to express an opinion on your point

HIEGER: Against the degradation theory is the fact that a low carcinogenicity has been shown by fats other than cholesterol, for example, olive oil, sesame oil, lard, wheat-germ oil, where the sterol content must be very low.

[illegible]

cancers simply because there is an abnormal milieu in the tissue. Dr. [illegible]

DOBRINER: I would like to mention an experiment that was done about 12 years ago and was never published. We were interested in the question of the carcinogenicity of aromatic amines. We obtained from one of the industrial plants very pure α-naphthylamine and β-naphthylamine. We injected these substances in the same sample of olive oil, obtained from one source. We didn't get any tumours with the β-naphthylamine but we got about 50 per cent of tumours with the α-naphthylamine. In control experiments, with the same olive oil we

REFERENCES

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DISCUSSION

SHOPPEE. I don't think I can add anything very significant to what Dr. Hieger has already said. There is this serious difficulty of the difference in susceptibility of individuals, even when large batches are used. I think one must agree that the evidence is conclusive that there is some substance, or substances, present in this widely distributed material, cholesterol, which is able to bring about neoplastic change, but as to the identity of that substance, there is no information at all.

GARDNER: How are the cholesterol, or the cholesterol derivatives, administered?

GARDNER: Has it ever been administered in the form of pellets? We have used cholesterol pellets in connection with hormone dilution experiments, and so far I have never seen a tumour around a cholesterol or a stilbœstrol-cholesterol pellet, although when injected in sesame oil, we have seen tumours

HIEGER. It is quite possible that the solvent plays a part in the formation of tumours. Lard itself gave no tumours when 350 control animals were injected. We were congratulating ourselves on this

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KELLIE: Is the so-called "commercial" cholesterol of animal origin?

HIEGER: Yes, from the spinal cord.

administered in the form of a solution in lard.

HIEGER: One has to consider not only the substance itself but the conditions under which it is present in the tissues. There is a lot of

tration of such a carcinogenic hydrocarbon. Weigert's experiments showed that benzpyrene was very rapidly metabolized either in liver or in skin.

SOMMERVILLE: It wouldn't be necessary to argue the formation of [illegible] experiments might be due to the different rates of absorption.

BEGG: Have you any evidence that steroids are destroyed in the tissues?

FOLLEY: There is evidence, which came from experiments with labelled acetate, that cholesterol is turned over in the udder; the cholesterol may be synthesized, and at the same time may be degraded in the lactating udder.

obtained about 60 per cent tumours. We still can't explain why the olive oil with β-naphthylamine didn't give any tumours.

HADDOW: What kind of tumours were they?

DOBRINER They were sarcomas.

HORNING: Did you ever attempt to transplant these tumours?

DOBRINER: No, nothing more was done.

DMOCHOWSKI. *I believe Dickens showed that a higher incidence of* tumours can be obtained by using mouse fat as a solvent. In view of the small amounts of active principle in your extract, Dr. Hieger, might not a more favourable solvent be helpful in obtaining a higher incidence?

HIEGER. Such experiments are in progress.

DMOCHOWSKI: Have any experiments been done on the comparison of activity of extracts from cancerous livers and normal livers?

HIEGER Steiner found in his first experiments in 1943 that cancerous livers were more potent than non-cancer livers, but five years later he had to reverse his opinion, and found that the difference was as great as nine to one. This is further evidence that the sensitivity of the animal is extremely important.

DOBRINER: I think he did the second experiment on a different strain.

HIEGER: I think he used a number of strains in each case.

DOBRINER It seems to be somewhat unwarranted to conclude that if one gets carcinogenic material from such drastically treated material, that the substances were there originally. Would you have any comment?

[illegible] Steiner does use a very drastic method for the saponification [illegible]

made with extracts simply prepared with benzene?

MUHLBOCK. I can confirm what Dr. Gardner said. I have treated more than 2,000 mice with cholesterol-œstrone pellets, but I have never [illegible] There is no [illegible] but [illegible] nted by sitosterol or stigmasterol.

BEGG: How long do you leave the pellets in?

MÜHLBOCK: One to two years.

BEGG: How big are these pellets?

MÜHLBOCK: One or two mg.

[illegible]

tumorigenic action of X-rays is apparently mediated through hormonal imbalances rather than by direct action upon the ovary (see Gardner, 1948*b*, for general statements and specific *schema*).

In our laboratory we have studied in considerable detail the responses of mice of seven different inbred strains, and of many hybrid groups, to prolonged treatment with œstrogenic hormone (Table I) (Gardner, 1948*a*).

Table I

TYPES OF TUMOURS APPEARING AMONG MICE OF DIFFERENT STRAINS WHEN SUBJECTED TO ŒSTROGENIC HORMONES*

Strain	*Mammary Tumours***	*Testicular Tumours*	*Lymphoid Tumours*	*Pituitary Tumours*	*Uterine Cervical Tumours*
A	+++	+++	−	−	++
C_3H	++++	−	+++	−	+++
CBA	+++	−	+++	−	+++
C_{57}	−	−	−	+++	+
JK	−	++	±	−	?
PM	−	−	++	±	++
$C_{12}I$	++	−	±	−	++

*Mammary tumours are the only tumours that occur spontaneously in mice of these strains and then only among female mice, whereas they appear in œstrogen-treated males Testicular or pituitary tumours have not been found in untreated controls Lymphoid tumours appear in a low percentage of mice of all strains and uterine cervical tumours only among mice of the PM stock

**++++ indicates a relatively high susceptibility and − no significant susceptibility to the tumours mentioned Reference +++, etc , indicates intermediate degrees of susceptibility.

In our experience mice of two strains, C_{57} and PM, acquire pituitary tumours subsequent to œstrogen treatment. This response is particularly consistent in animals of the C_{57} strain. In other strains such tumours either have never appeared, or have appeared with extreme rarity. The tumours are usually firm and fleshy, although occasionally cystic, and hyperplastic. They may become very large, almost as large as a normal mouse's brain. They are benign in that they never metastasize or infiltrate the meninges or brain. Histologically they are composed of chromophobic cells and have been designated chromophobe adenomas. The

THE EFFECT OF STEROID HORMONES ON EXPERIMENTAL PITUITARY AND GONADAL TUMORIGENESIS

W. U. GARDNER

In the presentation to follow it will be assumed that at least some of the steroid hormones, notably the œstrogens, are carcinogenic or tumorigenic. This is an assumption that may have to be modified in the future. In their carcinogenic activities these substances may act quite indirectly and through the intermediation of some factors or influences that at the present time, for the want of a more specific designation, are termed "inherited," "strain limited," or "species limited" factors.

Species and strain limitations in tumorigenic responses are also prevalent to some degree in responses to carcinogenic hydrocarbons, as well as other carcinogenic substances that may be somewhat less specific in their action than are the œstrogenic steroids. It is not our purpose here, however, to argue the point of whether or not the steroid hormones are carcinogenic in the same sense as are the carcinogenic hydrocarbons. Until more is known of the mechanism whereby these substances modify cellular physiology the discussion must border on the metaphysical.

The presentation to follow will be concerned with modifications of the steroid and pituitary-gonadotrophic and possibly other hormonal balances upon the incidence of pituitary and gonadal tumours in experimental animals, primarily mice. In some instances the œstrogens evoke tumours; in other instances, prevent them. Evidence will also be presented indicating the tumorigenic action of pituitary gonadotrophic hormone. Furthermore, evidence will be presented to show that at least so far as ovarian tumours are concerned the

tumorigenic action of X-rays is apparently mediated through hormonal imbalances rather than by direct action upon the ovary (see Gardner, 1948*b*, for general statements and specific *schema*).

In our laboratory we have studied in considerable detail the responses of mice of seven different inbred strains, and of many hybrid groups, to prolonged treatment with œstrogenic hormone (Table I) (Gardner, 1948*a*).

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In our experience mice of two strains, C_{57} and PM, acquire pituitary tumours subsequent to œstrogen treatment. This response is particularly consistent in animals of the C_{57} strain. In other strains such tumours either have never appeared, or have appeared with extreme rarity. The tumours are usually firm and fleshy, although occasionally cystic, and hyperplastic. They may become very large, almost as large as a normal mouse's brain. They are benign in that they never metastasize or infiltrate the meninges or brain. Histologically they are composed of chromophobic cells and have been designated chromophobe adenomas. The

cells composing them, however, are unlike any cells in the normal pituitary.

The pituitary glands of female mice are larger than those of males. The pituitary glands of mice of all strains hypertrophy somewhat subsequent to œstrogen treatment, but usually the amount of hypertrophy is limited. The normal mouse's pituitary weighs approximately 2 mg. Pituitaries weighing in excess of 12 mg. have been arbitrarily called adenomas. In some instances these glands are probably hypertrophied rather than adenomatous. Occasionally adenomas are found in smaller glands.

Mice of the C_{57} strain transmit the tendency to pituitary tumours to their F_1 hybrids (Gardner, 1948*a*). Backcrosses of the F_1 hybrids to parental strains indicate that the tendency for pituitary tumours to appear is transmitted as a dominant character (Gardner, 1941, 1948*a*).

How œstrogen acts in producing the chromophobe adenomas in the pituitaries of suitable animals is unknown. In many ways the hypertrophy resulting is comparable to that of the thyroid following the administration of thiouracil. The gonadotrophin and growth-hormone output by the glands is decreased. Evidence of modification of the thyrotrophic and adrenocorticotrophic hormones is not available. It can only be stated that in suitable animals such tumours occur.

The tumours appear at earlier ages in male than in female mice and usually attain larger sizes (Gardner, 1941). The simultaneous injection of testosterone propionate inhibits the formation of pituitary tumours.

The pituitary tumours grow subsequent to transplantation into animals of the same strain. So far, however, only about 60 per cent of the tumours that have been transplanted have grown, and then in only about 20 per cent of the animals into which they were transplanted. Furthermore, they have grown only in œstrogen-treated animals. When transplanted subcutaneously the growths first appear after approximately nine months and then continue progressively. Most of the

hosts have had, at the time that the subcutaneous transplants were made, tumours in their own pituitary glands. After the transplants have started to grow they apparently grow progressively in the absence of further hormone treatment.

An attempt has been made to determine whether or not the original lesions of the pituitary are reversible subsequent to cessation of œstrogen treatment. It is impossible to determine whether or not a pituitary tumour is present until it has reached a considerable size, in which case the skull may be misshapen. Two groups of mice were each divided into a control and an experimental group. In the control group œstrogen treatment was continued. The animals were over 400 days of age. In the experimental group œstrogen treatment was discontinued. The mice survived for appreciable periods of time in both groups and had pituitary tumours of comparable sizes. From these observations it can be concluded that at least the pituitary tumours do not regress subsequent to the discontinuation of œstrogen treatment. It is quite possible that they continue to grow.

Ovarian Tumours

Ovarian tumours have been induced in experimental mice by two methods: (1) the intrasplenic transplantation of ovaries into castrated animals (Li and Gardner 1947*a*, 1947*b*, 1949) and (2) irradiation with adequate doses of X-rays (Furth and Butterworth, 1936). About three years ago the hypothesis was made that ovarian tumours in castrated animals bearing intrasplenic transplants arose because of the hormonal imbalance existing (Gardner, 1948*b*). The livers of the mice destroy œstrogens (Rush, in press). Zondek has first shown, about 17 years ago, that the rat's liver destroyed œstrogens. Subsequent to gonadectomy the amount of gonadotrophic hormone in the pituitary is increased (Engle, 1929, Evans and Simpson, 1929). Thus an ovary placed in the spleen, pancreas or other sites drained by the portal vein has its hormones destroyed by the liver before they reach the systemic circulation. In such animals

the pituitary gonadotrophin is elevated (Fig. 1). In such mice ovarian tumours of the granulosa cell type develop almost invariably. So far in our experience the tumours have not been strain-limited. Animals of six different inbred strains and many hybrid groups have acquired such tumours.

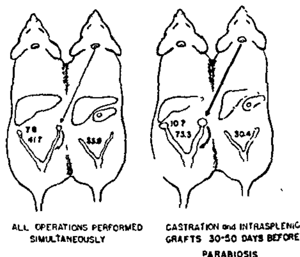


Fig. 1.
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gonadotrophins. This observation extends experiments undertaken by Dr. Li, in which he showed, by direct pituitary assay, that gonadotrophins were increased in animals with intrasplenic ovarian transplants.

The tumours first appear approximately six months after intrasplenic transplantation, although others appear after approximately 300 to 400 days.

Ovarian tumours have not appeared in intrasplenic transplants in animals that have acquired adhesions of the transplants of the parietal peritoneum, in animals that have

had one ovary or testis in place, or in animals that received œstradiol or stilbœstrol or testosterone propionate in adequate amounts (Li and Gardner, 1947*a*, 1947*b*, 1949).

The tumours are predominantly granulosa cell tumours although luteomas, which I believe should be called luteinized granulosa cell tumours, are not infrequent. Many of the tumours produce active substances of œstrogenic or androgenic type in such amount or of such quality that they by-pass the liver and produce effects upon the accessory reproductive organs of the host. If the tumours are permitted to grow to a large size the incidence of hepatic extension and pulmonary metastases becomes quite high. The tumours grow, in most instances, subsequent to transplantation into the subcutaneous tissues of hosts of the same strain. The percentage of takes and the rate of growth differ with different tumours.

Hypervolæmia was observed by Dr. Furth in animals bearing transplanted ovarian tumours (Furth and Sobel, 1947). A similar condition has been seen in our laboratory and has been studied, especially by Dr. Wolstenholme. The liver and adrenal glands may be increased in size several times by dilatation of the sinuses. The blood volume was greatly increased. Dr. Wolstenholme (1950) has demonstrated that this condition is reversible, subsequent to the removal of the tumour, and that the active substance will not by-pass a parabiotic union. The cause of hypervolæmia cannot be associated with the ability of a tumour to produce any of the known hormones. It is rarely found in animals having other than gonadal tumours.

One might ask why do not granulosa cell tumours occur more frequently in ageing animals? In advancing age the ova and follicles are depleted and presumably, also the hormone-producing capacity of the ovary. In such circumstances as after the menopause the ovary should be in an environment of augmented gonadotrophic hormone. In collaboration with Dr. Li (Li and Gardner, 1950) experiments were undertaken in which young ovaries were transplanted into old mice and old ovaries transplanted into old mice and

old ovaries were transplanted into young mice. Few tumours occurred in the intrasplenic transplant in the castrated mice of the first two groups but old ovaries transplanted into young mice became tumorous as frequently as did young ovaries transplanted into young mice. It thus seems that the ageing organism does not produce an environment as conducive to ovarian tumorigenesis as does the younger organism. Dr. Fern Smith in our laboratory (Smith and Gardner, 1949) has observed that the level of gonadotrophic hormone, as determined by the capacity of the pituitary to produce uterine stimulation in young assay animals, is decreased with advancing age after the age of 400–500 days. It is thus probable that gonadotrophic hormone production decreases with advancing age more or less in relation to the decrease in ovarian function.

Ovarian tumours also occur subsequent to X-irradiation. In preliminary experiments undertaken in collaboration with Dr. Li and Dr. Kaplan it was found that the injection of testosterone propionate in doses of 1·25 µg. weekly and œstradiol benzoate prevented the appearance of such tumours. More recent experiments, however, in which mice of the BC strain have been used, indicate the testosterone propionate in the amount administered does not prevent ovarian tumours; in fact the incidence has been somewhat higher than in the irradiated mice given sesame oil (Gardner, 1950) (Figs. 2 and 3). This, however, is in part due to the fact that testosterone propionate inhibits the leukæmogenic action of X-rays (Fig. 3). Œstradiol benzoate, however, completely prevented the appearance of ovarian tumours in irradiated animals, and also prevented the pre-tumourous changes observed subsequent to X-irradiation (Fig. 4). Experiments under way indicate that larger amounts of testosterone propionate will also prevent ovarian tumorigenesis in mice of the BC stock.

Testicular Tumours

Interstitial cell tumours of the testis appear in mice of the A strain subsequent to the injection of œstrone, œstradiol or

its esters, and stilbœstrol (Gardner, 1948b). Such observations have been made in several laboratories in England and

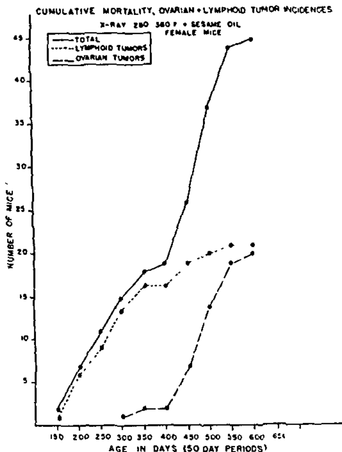


FIG. 2. Female mice of the B.C. stock (12th–15th generation of inbreeding) that received total body irradiation and weekly injections of 0·05 ml. of sesame oil. The number of lymphoid and ovarian granulosa cell tumours are indicated

America. One other strain, strain C, interestingly one of the parental strains from which Dr. Strong derived the original

A strain, also acquire such tumours subsequent to œstrogen treatment. In our experience testicular tumours have

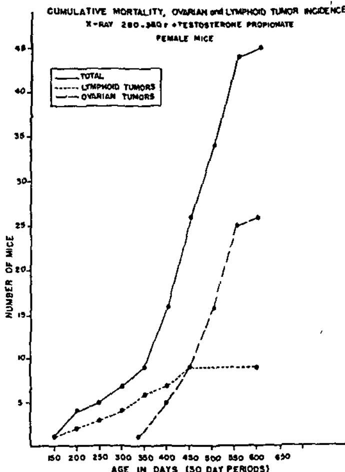
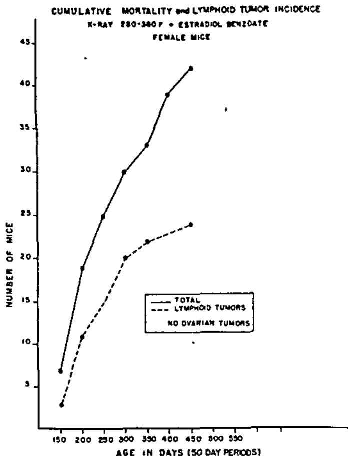


FIG. 3. Mice of the BC stock (12th–15th generation of inbreed- [illegible]

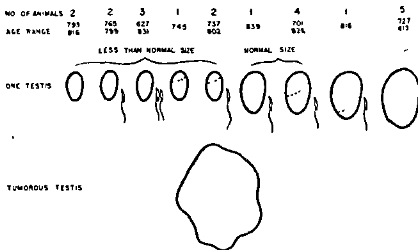
occurred with extreme rarity in œstrogen-treated mice of other than the A strain, or in untreated mice of any strain.

t has been assumed, but is as yet unproven, that in mice of he A strain the administration of œstrogenic hormones



results in an increase of pituitary luteinizing hormone or nterstitial cell-stimulating hormone.

Recently it has been found that mice of one group, CC_1, which never acquire interstitial cell tumours subsequent to the injection of other œstrogens (Gardner, 1941) did so subsequent to prolonged treatment with tri-*p*-anisyl chloroethylene (TACE). Forty-six of 92 animals treated weekly with 50 or 100 μg. of this synthetic œstrogen had testicular interstitial cell tumours at death (Fig. 5). It is hoped that



the above observation may provide a method for determining how œstrogens may act in inciting interstitial cell tumours.

Summary

The tendency for pituitary tumours to occur in œstrogen-treated mice is strain-limited and is transmitted by both male and female mice of the C_{57} strain to their first generation hybrids. The tumours are not completely autonomous, in that they grow only, and then not consistently, in œstrogen-treated mice of the same strain Prolonged quiescent periods have always preceded the growth of the tumours.

Ovarian grafts transplanted to sites drained by the portal circulation in castrated hosts give rise to granulosa cell tumours. These tumours can be prevented by the injection of œstradiol benzoate or testosterone propionate. Ovarian tumours occurring after irradiation (X-rays) can also be prevented by the injection of œstrogen. Ovarian tumours arising under both conditions are probably attributable to the gonadotrophic hormones produced.

Testicular interstitial cell tumours occur in œstrogen-treated mice of the A strain. Again, the tendency for such tumours is transmitted by both males and females to the first generation hybrids. A synthetic œstrogen, tri-*p*-anisyl chloroethylene, has induced tumours in mice ($C_{57} \times$CBA) previously not showing such tumours.

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DISCUSSION

PULLINGER. In the intrasplenic ovarian tumour experiment, can you transplant those tumours into intact animals? And in the pituitary tumour experiments, can you transplant the tumours into castrated animals? That would show complete independence of hormone in the two cases?

GARDNER. We have not attempted to transplant all of the ovarian tumours, but about 60 per cent of the tumours we have tried to transplant have grown subsequent to transplantation into intact animals. The incidence of take at the first transfer generation is variable. With some of the tumours there was evidence that progesterone increased the percentage of takes and the rate of growth. That has been published by Dr. Pan and Dr. Cliffton. After the first transfer, however, most of these tumours will grow in almost 100 per cent of the animals into which they are transplanted, whether male or female, or castrated.

I have not tabulated all the data, but the percentage of "takes" is high. As for the pituitary tumours, we have transplanted them into castrates, into normal males and females, and into œstrogen treated males and females. The only "takes" we have seen so far have been in the œstrogen treated animals, and even among those there has been a comparatively low incidence of tumours providing "takes," and a comparatively low number of "takes" with any one tumour transfer. We have not been able to carry any of them beyond the fourth transfer generation, that is, a span of six years.

MÜHLBOCK: In answer to Dr. Pullinger's question, I have been study- [illegible] but it requires a longer time in the castrated animals than in normal males.

[illegible] much of their specificity for the hormonal environment in which they develop. I think that the hormone environment which is best for their growth varies from tumour to tumour.

MÜHLBOCK: The principal cause for the development of all these [illegible]

the pituitary using immature animals. Dr. Fern Smith did some experiments on the age change in the pituitary, using both castrated and intact mice. She found that after 500 days the gonadotrophic hormone content of the pituitary decreased. She took pituitaries from mice from 300, 400, 500, 600 to 700 days, macerated them, injected them subcutaneously into infantile mice, and then weighed the uteri of the assay animals. Castrated immature animals were used as controls. The stimulation of the uterus is indirectly through the ovary and hence gonadotrophic hormone content is maximum at around 500 days, and decreases thereafter. Dr. Miller in our department put in parabiosis a pair of animals, one of which was castrated 30–50 days before the parabiotic union, and weighed the ovaries and the uteri of the intact parabiont after 14–21 days; the uterus weighed about 73·3 mg. Then he did the same thing with animals with an intrasplenic ovarian transplant, with the same effect. The intrasplenic ovarian transplant did not produce enough hormone to prevent the pituitary of the castrated parabiont with the intrasplenic ovarian graft from producing an abnormally large amount of gonadotrophin, stimulating the ovaries and hence the uteri of the intact parabiont. When Miller [illegible]

30–50 days the gonadotrophic hormone was high enough to produce a sudden effect in the intact parabiont. The same experiment is being done using animals with irradiated ovaries.

MUHLBOCK: There must be a difference between rats and mice, because we know that in rats there is an increasing amount of follicular [illegible]

[illegible]

very big ovaries but there are no tumours.

GARDNER. Have you put them in parabiosis with males?

MÜHLBOCK: Yes, with males and females of pure strain mice and hybrids. The females which are very susceptible to œstrone production died, because of retention of the bladder and nephrosis indicating that there must be a great amount of œstrogenic hormone production. After parabiosis of several months, sections of the ovaries show the same

picture you find after the injection of the luteinizing hormone; very

it indicates tl
treacherous tc
not proven.
and FSH differentially.

MUHLBOCK The strain of mice is important for the development of

like to show.
[not reproduced]. Would you call that a tumour?

old mice, and we have studied a fairly large number of animals.

liver would inactivate
the livers of mice that
were subjected to chronic œstrogen treatment and castrated as well showed less activity, about 6 5 μg. per mg. per hour. I have often wondered whether the occasional mouse that shows these minimal ovarian changes might really have some deficiency in the œstrogen destroying mechanism.

DMOCHOWSKI In which strain did testosterone have an inhibitory effect on lymphoid tumours?

GARDNER: The data presented today were for mice of the BC strain, and that has since been confirmed in another series of animals. We find the same thing in both the males and females, but I showed only the data on the females. Earlier experiments on œstrogen treated

mphoid tumours in

done only intact males and females.

DMOCHOWSKI. Is there a difference in the incidence between females and males?

GARDNER: Not in this strain. It is interesting, because in general, female mice show a higher incidence of leukæmia than male mice. The incidence in this strain is only about 3 per cent.

DMOCHOWSKI: Yet, in spite of that, testosterone seems to exert an inhibitory effect?

GARDNER: Yes, and the normal testis will largely prevent the increased incidence of leukæmia after irradiation. The incidence in the sesame oil treated X-rayed females was, as I recall, 53 per cent; the incidence in the sesame oil treated X-rayed males was 14 per cent, just the same as that of X-rayed females that got testosterone, so the testes are just as effective as 1·25 mg. testosterone propionate in

tumours.

The incidence, with large doses of œstrogen, goes up to about 25 per cent, whereas in the control it was 1–3 per cent. If we

KORTEWEG: You have shown that the possibility of growing pituitary tumours probably has something to do with hormones, and is also genetically determined. Did you find any correlation between this probable hormone factor and the genetically determined hormone

GARDNER That's a very important point that I have thought about a great deal In my experience these tumours have occurred frequently in animals of what we call the BC, a low mammary tumour strain,

If reciprocal hybridization is done, most of the animals will develop mammary tumours and pituitary tumours. So far as our own observations are concerned, I would think that any possible relationship with respect to the high incidence of pituitary and mammary tumours

after œstrogen treatment is just incidental. I have not, however, seen the tumours in the R-III animals, which are susceptible to mammary tumours.

KORTEWEG: Did your CBAs with tumours have the milk influence?

GARDNER: Yes. Our CBAs are just like the C_3Hs in this respect.

KORTEWEG. Then we can understand the fact that in the F_1 $C_{57} \times$ CBA, there are no mammary tumours, and in the reciprocals there are mammary tumours. Did you find that there was a positive correlation between the presence of the genetically determined hormonal factor and the appearance of tumours of the pituitary gland and of other organs?

GARDNER: Of course, we couldn't get results when we had the milk influence there because the mammary tumours developed so early that the animals didn't live long enough. I showed a slide [not reproduced] of one experiment in which we hybridized mice of the C_3H strain

PART II

THE MAMMARY GLAND

SOME EFFECTS OF STEROIDS ON THE MAMMARY GLAND

S. J. FOLLEY

I do not work in the tumour field, but Professor Haddow asked me as an endocrinologist specially interested in lactation to contribute to this symposium in view of the outstanding importance of the mammary gland in cancer research. I thought that perhaps he would like me to give some general account of the effects of steroids on mammary growth, with special reference to the work that we have been doing in our laboratory at Reading.

Methods for Studying the Growth and Development of the Mammary Gland

First of all, I think one should emphasize the urgent need for some objective methods for studying the effect of hormones on mammary growth (see Richardson, 1947). For many years there has been a tendency among workers in this field to regard the study of only one mammary gland from each of a group of hormone-treated animals, either in the form of whole mounts or sections or sometimes both, as a sufficient basis for drawing rather far-reaching conclusions about the effect of various hormone treatments on the structure of the mammary gland. This is admittedly a time and labour saving procedure but its inadequacies may easily lead one astray. In the last few years we have, in our laboratory,

been trying to develop more objective methods for studying mammary growth, and our aim has been to make them as quantitative as possible. It should be emphasized, however, that this is proving rather difficult with the mammary gland. I shall begin by giving a brief account of the methods which we have been using, and which we hope continually to improve, for studying mammary development under experimental conditions. I hope that these methods may be of interest and perhaps of some use to cancer research workers.

In species such as the mouse, rat and monkey, in which the mammary glands are relatively flat (flat, that is, in all reproductive phases except late pregnancy and lactation) and can be stripped off in a thin sheet of connective tissue and made up into whole mounts, one objective measure of mammary growth is given by the rate of increase in the area of the mammary gland (bounded by the shortest line joining the periphery of the duct system). We have recently been carrying out studies involving measurements of the total area of all the mammary glands of the rat. Whole mounts are prepared, their images projected on to squared paper and their outlines traced. In this way the total mammary gland area for each animal can be computed. It should be emphasized that measurement of the mammary gland area, though affording an objective and quantitative method for studying the dynamics of mammary growth, only gives a general idea of the rate of growth of the duct system. It gives no information about morphological changes occurring within the periphery; it fails to detect increases in the degree of arborescence of the ducts, or the formation of new alveoli.

The most suitable and usual type of animal for studying experimental mammary growth is the immature animal which has been gonadectomized when sexually immature. Such an animal is, of course, still growing during the experimental period, and in studying the rate of increase of mammary area one has to take into account the concomitant increase in the body surface. This can be done by means of relative growth analysis (see Huxley and Teissier, 1938) which

enables a comparison to be made between the rate of growth of the mammary gland and that of the body surface.

The law of simple allometric growth of Huxley and Teissier, $y = bx^{\alpha}$ (where y is the size of the organ under consideration, x the size of the reference organ—often the body as a whole—and b and α are constants), may be written $\log y = \alpha \log x + \log b$. So that if the law is obeyed, a plot of log y against log x gives a straight line the slope of which is α, the equilibrium constant. If α is greater than 1, the organ is growing faster than the body (positive allometry), which is to be expected if it is a target organ responding to its specific hormone. If $\alpha = 1$, then the organ is merely growing at the *same rate as the body as a whole (isometry), and any observed* increase in the size of the organ must be discounted. This method of analysis was, I think, first applied to the mammary gland by Professor Zuckerman and myself in studies on the breast of the rhesus monkey some years ago (Folley, Guthkelch and Zuckerman, 1939). It has also been used in France by Dubois (1944) in some unfinished work which was unfortunately interrupted by his untimely death, and has been used in our laboratory more recently in studies on the mammary gland of the rat (Cowie, 1949).

Relative growth analysis is also very suitable for studying the dynamics of teat growth since the length of the teat can be readily measured and compared with the length of the body. As an example I might mention some work carried out in our laboratory some years ago, on the effect of androgens on the growth rate of the guinea-pig teat; this work showed that unsaturated androgens such as testosterone and androstenediol caused the teat to grow allometrically while saturated androgens such as *cis*-androsterone and dihydrotestosterone had no effect (Bottomley and Folley, 1938). Another example of the use of this method in teat growth studies is provided by our work on the normal and œstrogen-induced growth of the teat in the virgin female goat (Folley, Scott Watson and Bottomley, 1941). The teat of the normal virgin female goat begins to grow allometrically quite soon after

birth. The allometric phase is, however, eventually superseded by a period when the teat ceases to grow, and this occurs, curiously enough, just when the œstrous cycles of the goat's first breeding season begin. At the end of the breeding season, when the œstrous cycles cease, allometric growth, however, sets in once more. This curious phenomenon, which is the exact opposite of what one would expect at first sight, would clearly repay further investigation.

The mammary gland also grows by densification of the duct tree and develops by the differentiation of lobule-alveolar tissue. Therefore, in addition to the use of relative growth analysis in conjunction with measurements of mammary area, we have attempted to devise methods of obtaining objective measures of the increase in the arborescence of the mammary duct system and of the formation of alveoli. This problem has been attacked by devising a scoring system, applied to mammary gland whole mounts, which gives numerical scores susceptible of statistical analysis, thus providing a semi-quantitative if still somewhat subjective measurement of mammary development (Cowie and Folley, 1947). Dr. Dora Jacobsohn of Lund has also used our method, apparently with success (Jacobsohn, 1948).

The features which we score according to a pre-determined subjective scale are: the degree of arborescence of the duct system, the presence or otherwise of club-shaped end-buds (which usually take stain deeply and are considered to be a sign of active duct growth), and the status of the gland regarding the differentiation of side-buds and alveoli. The total whole mounts from each group of rats in an experiment are randomized and scored independently by two observers, the mean score for each feature being calculated for each group. The results can be submitted to an analysis of variance, thereby affording, we believe, a more valid picture of hormonal responses than if one gland only from each rat is studied microscopically in the usual way.

The third method I wish to describe is applicable to species that have "three-dimensional" mammary glands, i.e. udders

or breasts. It has been developed in collaboration with Mr. K. C. Richardson of the Anatomy Department, University College, London, who has been associated with us in these studies for a number of years. It has been used by us solely in studies of the goat udder, but in theory it could be used not only for the udder of the cow (though technical difficulties in working on such a relatively huge mass of tissue would be rather great) but presumably also for the human breast. In studying the structure of the caprine or bovine udder, it is very little use excising a small piece of tissue and fixing and sectioning it in the usual way, because it is apt to be full of milk, and as soon as the udder is cut the milk drains out and the whole organ collapses. The collapse of the distended alveoli precludes the possibility of getting a true picture of structural relationships. Moreover, in our experience, experimentally developed udders in the goat present a far from uniform histological picture so that a small piece of tissue is unlikely to be a representative sample.

In applying the method devised by Mr. Richardson, my colleague, Dr. A. T. Cowie, removes one half of the udder (one gland) from an anæsthetized goat—rather an exacting operation since the slightest puncture of the gland cistern is precluded otherwise the whole organ collapses—and as soon as it is taken off it is perfused with fixative under pressure. Later, in Mr. Richardson's laboratory, it is sliced in a horizontal plane into slices about 1·5 cm. thick, and fixing is continued. The slabs are finally infiltrated with collodion, and sections cut at 100 μ are stained and mounted as whole sections of the entire gland. The sections can be studied by suitable statistical sampling methods for "porosity" (the number of alveolar spaces per unit area, which is related to the mean alveolar diameter), and also examined microscopically.

With the help and advice of Mr. Richardson, an essentially similar method has also been applied by Dr. Cowie and Dr. M. H. I. Macaulay to the guinea-pig, which we have used as a pilot animal for the goat. The reasons for the choice of the

guinea-pig as pilot animal are (*a*) that its two mammary glands are "three-dimensional" of a form rather like udders in miniature, and (*b*) like the goat udder, the guinea-pig mammary gland responds with extensive alveolar development to the action of œstrogen alone. The whole animal is fixed under anæsthesia by perfusion through the aorta, the mammæ removed and serial sections prepared after collodion infiltration.

Mammogenic Effects of Œstrogen and Progesterone

In turning to consideration of our studies of the effects of steroids on mammary growth, it should first be emphasized that the common species of experimental animal can be divided into two main classes. First, there are those in which œstrogen alone evokes not only growth of the mammary ducts, but also considerable alveolar development. These *include the guinea-pig, the monkey and the farm ruminants.* The case of the rhesus monkey is of some interest. Earlier work (Folley *et al.*, 1939; Gardner, 1941) did not lead to unanimous agreement as to how far œstrogen alone would cause development of the mammary alveoli in the rhesus monkey, particularly the male. However, the recent valuable monograph of Speert (1948), who has had access to much more comprehensive experimental material than earlier workers, leaves no doubt that the monkey must be included in the species in which œstrogen alone causes extensive lobule-alveolar growth. As regards the ruminants, the cow and goat, in which we ourselves are mainly interested, numerous experiments on the hormonal induction of lactation (reviewed by Malpress, 1947) testify to the extensive alveolar development which can be obtained by œstrogen treatment. Fig. 1 shows a complete section at 100 μ. of one half of the udder of a virgin goat which had been given 1·0 mg. hexœstrol daily for 96 days, that is a period about equal to one half of the gestation period (Cowie, Folley and Richardson, unpublished). *The extensive lobule-alveolar system developed by* this treatment is shown very clearly. However, in considering

experiments which appear to demonstrate alveolar growth in response to œstrogen in the absence of the ovary, there is a complication to which I drew attention ten years ago (Folley, 1940), namely, the possibility that, under the influence of administered œstrogen, the adrenal cortex may secrete progesterone which, as is well known, is a mammogenic agent the action of which is closely related to the growth of alveoli. There seems to be no obvious way of overcoming this technical difficulty.

The second category, which includes the mouse, rat and rabbit, consists of forms in which, at any rate at physiological dosage levels, extensive alveolar growth requires progesterone as well as œstrogen. The position as far as the rabbit is concerned is very beautifully illustrated by the elegant work which Dr. Lyons has done on the optimal ratios of œstrogen and progesterone necessary for the experimental development of the lobule-alveolar system (Lyons and McGinty, 1941; Scharf and Lyons, 1941).

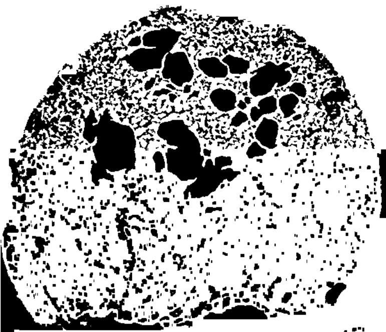
Goat. In our earlier studies on experimental udder growth in the goat, the mediocre lactational performances of the experimentally developed udders quite soon led us to suspect that although extensive udder growth regularly resulted from the administration of synthetic œstrogens alone, the alveolar tissue so obtained might not only be deficient in amount but might also exhibit morphological abnormalities, both of which features could perhaps be corrected if progesterone were administered as well. Indeed, Mixner and Turner (1943) in the United States have shown, in preliminary experiments with the goat, that udders grown with œstrogen tended to exhibit cystic alveoli in which papillomatous epithelial outgrowths were often seen, but if progesterone was given as well, the alveoli were smaller and the histology of the tissue more nearly normal In view of these considerations we have been investigating for some time the use of various ratios and absolute levels of œstrogen and progesterone for evoking artificial udder growth in spayed goats. So far only a brief, preliminary account of this work, which is still in progress,

has been published (Cowie, Folley, Malpress and Richardson, 1951).

In choosing the most likely gravimetric ratio for the best results we took into consideration the work of Dr. Lyons, just mentioned, in which he investigated the optimal ratios in the rabbit. On the basis of his results and using Dr. Corner's calculations of the output of progesterone by the rabbit's corpus luteum (Corner, 1937), and our own measurements of the size of the goat corpus luteum (Folley, Greenbaum and Roy, 1949), we calculated that daily doses of 40 mg. of progesterone and 1 mg. of hexœstrol were worth trying first in our search for an experimental régime capable of mimicking in the spayed goat the effect of pregnancy on the udder of the *normal female. The hormones were injected in oil daily* for 20 weeks (approximately the gestation period in the goat) and, since mammary growth largely takes place during the first half of pregnancy, some animals were injected for 10 weeks only.

In preliminary experiments the indications were that combined treatment with œstrogen and progesterone developed alveolar tissue histologically more normal than that obtained in response to œstrogen alone. However, subsequent experiments on a larger scale did not always bear out this indication though the results suggested that the combined treatment grew udders often capable of giving larger milk yields than udders grown with œstrogen alone. Alveolar "porosity" measurements made on sections prepared from udder halves removed from these animals at the peak of lactation were very variable and no statistically significant differences were ascribable to the different treatments.

For various reasons it seemed possible that the œstrogen doses used in these experiments were too high and that more significant results might be obtained with lower dosage levels. In this connection it may be noted that Clarke and Selye (1943) have reported that a given ratio of œstrogen and progesterone will evoke different responses in the vaginal epithelium of the rat according to the absolute level at which



[illegible]

by Mr R C Richardson

Photograph by Mr F J Pittock, F R P S.

[*To face page* 76



FIG 2 Celloidin sections of mammary glands of ovariectomized virgin guinea-pigs. The sections, shown all at the same magnification, were cut at 30 μ. and stained with hæmatoxylin

Top left	Untreated control
Top right	Guinea pig receiving 0 1 mg œstrone subcutaneously in oil daily for 68 days
Bottom left	Guinea pig receiving 2 4 mg progesterone in oil daily for 68 days
Bottom right	Guinea-pig receiving 0 1 mg œstrone and 2 4 mg progesterone subcutaneously in oil daily for 68 days

Photographs by .

two hormones are given, vaginal cornification resulting at : level of dosage, and an entirely different response at a her level. It seemed possible that the same might apply growth responses of the mammary gland. We are therefore rying out at the present time a preliminary experiment on r spayed goats in which a much lower dose of œstrogen 25 mg. daily) is being given, but at the same time we have sed the progesterone/œstrogen ratio to 400.1 by weight.

A very interesting effect has been observed in these latest periments. The two goats receiving hexœstrol alone, vards the end of the injection period of 20 weeks developed narkably large udders—as large as those of high-yielding digree goats in full milk. These udders were so turgid as necessitate the initiation of milking before the end of the atment period. By contrast the udder development in the o goats receiving œstrogen and progesterone was much less iking. At first sight it appears as if the high daily dose of ogestero [illegible] the reolar t [illegible] tain til the rather laborious histological studies on the udder lves removed at the peak of lactation are completed by :. Richardson. It is worth noting that Dr. Lyons found idence of inhibition of alveolar growth by high progesterone/ strogen ratios (just over 600:1) in his rabbits (Lyons and cGinty, 1951).

As regards the effect of steroids on the male mammary and, though in many species, well known examples of which e the guinea-pig and the monkey, the male gland is equipontial with the female gland, our experience as well as that others indicates that this is not so in the ruminant. In our periments, castrated male goats subjected to prolonged strogen treatment have never shown any but the most

*[illegible]

restricted mammary development, the experimentally grown glands never extending beyond the base of the nipple, though containing some alveoli (Folley *et al.*, 1941). Dr. Malpress and I in similar unpublished experiments on steers, to which we gave œstrogen and progesterone, obtained very similar results (see Malpress, 1947).

Guinea-pig. Our experiments on the hormonal induction of udder growth and lactation in goats which are being carried out with a view to eventual practical application, are very expensive in animals and hormones. As was stated earlier we are at the same time investigating the optimal progesterone/œstrone ratios and levels for mammary alveolar development in a much less expensive pilot animal, the guinea-pig. In experiments involving the administration of various daily doses of progesterone and œstrone to spayed virgin guinea-pigs, set up according to a factorial design (for which we are indebted to Prof. C. W. Emmens), Dr. Cowie has found that in the guinea-pig, progesterone is necessary in addition to œstrogen for normal and full alveolar development. The type of result obtained is illustrated in Fig. 2. The dosage ranges, given daily for 68 days (the gestation period) for the best responses have been narrowed down to 50–100 μg. œstrone and 0·5–4·0 mg. progesterone and these ranges are being studied more closely. When the optimal progesterone/œstrone ratio for mammary growth in the guinea-pig has been worked out it is intended to try this on the goat.

Rat. I would like now to consider some experiments on the rat, which, along with the mouse, has been widely used for studying effects of steroids on mammary development. We have applied relative growth analysis to study the rate [illegible] normal animal and [illegible]

As [illegible] obtained from a plot of the mammary area against age in young animals is limited. Such curves, for instance, give no information about the age at which the ovary first begins to secrete enough œstrogen to change the isometrically growing

gland to an allometrically growing one. Using relative growth analysis, however, Dr. Cowie has shown that in female Norway rats from our colony the mammæ grow isometrically from birth until 21–22 days, when an allometric phase sets in, the constant a assuming a value of approximately 3. It therefore appears that the ovary first begins to secrete enough œstrogen to affect the growth of the mammary gland at 21–22 days. If the females are spayed at 21 days, isometric growth ($a=1$) continues (Cowie, 1949).

Mrs. M. Silver, in our laboratory, has been investigating the effect on the equilibrium constant, a, of various dosages of œstrogen given to spayed females. The immediate object was to find out whether it is possible, by the administration of sufficient œstrogen, to induce faster relative mammary growth than is observed in normal females ($a=3$). Some of the results obtained so far are shown in Fig. 8.

The results so far obtained show that with doses of 0·25 μg. œstradiol dipropionate every two days a value of a of about the same magnitude as that observed for the intact female is obtained. Increasing the dose to 1·0 μg. every other day did not increase a significantly. It therefore seems that excess of œstrogen does not cause the mammary ducts to grow much faster than the normal rate, which may represent about the maximum growth rate attainable. When we have determined the minimum dose of œstrogen which just gives an a value of 3, it may be that we would be justified in thinking that we have an upper limit for the œstrogen output of the rat ovary. In these experiments the value of a found for untreated, spayed controls was 1·3, which is somewhat higher than the value of unity required for isometric growth and which was obtained in previous experiments with rats from the same colony (Cowie, 1949). It is believed that this discrepancy is connected with the fact that in these experiments the animals were spayed at 10 days, whereas in the previous experiments of Dr Cowie they were spayed at 22 days, i.e. immediately before observations started. It may be that in the interval of 11 days between removal of the ovaries and

the start of observations in the present experiments, there was some activation of the adrenal cortex by a hypophysis released from the control of the ovary, resulting in the production of small amounts of mammogenic steroids, perhaps

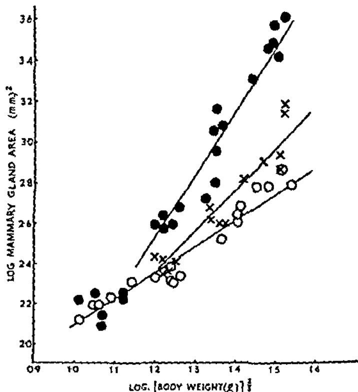


FIG. 3. Effect of œstrogen on the relative rate of increase in total mammary gland area in the young female rat.

● Normal intact females receiving 0·1 ml Arachis oil subcutaneously on alternate days.
○ Spayed females receiving 0·1 ml Arachis oil subcutaneously on alternate days.
× Spayed females receiving 0·05 μg. œstradiol dipropionate in 0·1 ml. Arachis oil subcutaneously on alternate days.

œstrogen. A more clear-cut, because longer term, example of this phenomenon was described by Fekete, Woolley and Little (1941). In support of this interpretation, Mrs. Silver

has found that in these early spayed animals the adrenals at autopsy were slightly heavier than normal for their age.

Similar results were obtained with male rats. Like the females, males gonadectomized at 10 days and killed at intervals from 21 days on, gave an α value somewhat greater than unity and the explanation is probably the same as for females. Administration of various doses of œstrogen gave much the same results as in the female rats.

This series of experiments has also included an investigation of the effect of aminopterin, a folic acid antagonist, on mammary growth as studied by this method of analysis. Hertz and Tullner (1949) have shown that administration of aminopterin to the spayed female rat virtually abolishes the uterine weight increase evoked by œstrogen treatment and have concluded that folic acid plays a part in this œstrogen-induced response. We therefore felt it to be of interest to see if there was any evidence that folic acid is concerned in mammary duct growth responses to œstrogen. However, we have so far been unable to demonstrate any inhibitory action of aminopterin on mammary growth, either in the normal intact female during the allometric phase or in the spayed animal receiving just sufficient œstradiol to cause allometric mammary growth.

Mammogenic Effects of Androgen

Rat. Coming now to the effects of androgens on the rat mammary gland as shown by relative growth analysis, it has been found that the mammary gland grows isometrically in intact males and that castration does not alter the α value, indicating that androgen at any rate at endogenous levels has little or no effect on the growth of the mammary ducts (Cowie, 1949). A study of whole mounts, however, illustrates the limitations of this analysis because whole mounts show that androgenic factors indeed have a remarkable effect on the mammary gland of the male rat In confirmation of the findings of earlier workers (e.g. Astwood, Geschickter and Rausch, 1937), Dr. Cowie has found that though the mammary

duct system in the young male rat remains restricted there is a striking development of clusters of alveoli at some time between 30 and 60 days. The mammary gland of the intact adult male rat thus has quite a characteristic structure; the duct system is restricted in extent but is covered with dense clusters of alveoli.

Rhesus Monkey. Just as in the case of the response of the mammary rudiment to œstrogen, so with androgen, species differences must be taken into consideration. This is exemplified by the case of the rhesus monkey. In experiments which I carried out with Dr. Van Wagenen at Yale some years ago we found that in the spayed female monkey androgen would only cause alveolar development if alveoli were already present (Van Wagenen and Folley, 1939). In these circumstances testosterone propionate was capable of causing the development of dense alveolar tissue while androsterone was inactive, recalling the effects, mentioned earlier, of these two androgens on the guinea-pig teat. Another observation which we made, confirming effects which Professor Zuckerman and I had described earlier (Folley *et al.*, 1939), was that papillomatous projections of the alveolar epithelium into the lumen were often present after androgen treatment in the monkey. In the monkey we have not succeeded in demonstrating growth of the duct system in response to androgen; the only noticeable effect was that the ducts were dilated with secretion. On the other hand it is well known that in the mouse administered androgen does cause duct growth (Van Heuverswyn, Folley and Gardner, 1939).

Since this is a conference on tumours, I feel I ought to conclude with a few words about cancer. In our work on the monkey mammary gland, Professor Zuckerman and I described some animals which had received regular treatment with natural œstrogens for periods ranging from over 365 to 938 days (Folley *et al.*, 1939). Although considered in relation to the normal life-span, this period of time is small in comparison with the period for which œstrogen is commonly administered to mice in order to induce mammary cancer,

it may be noted that we never saw any neoplastic or other abnormal conditions in these mammary glands when the animals were finally brought to autopsy. We felt at the time that this might provide some reassurance for the clinical use of œstrogen in women.

For the gift of material used in the unpublished experiments described in [illegible]
W [illegible]
C. [illegible]
and to Dr. J. H. Williams of the Lederle Laboratories Division, American Cyanamid Co. for aminopterin.

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DISCUSSION

LYONS: I do not think anybody would thank me for dragging in the hormones of the anterior pituitary at this time in connection with steroid function; but they do play very important roles in determining the response of the mammary gland to the ovarian steroids.

PULLINGER: I have often thought, like Dr Folley, that the mammary gland response might finally tell us the physiological level of œstrogen in these animals. I take it there is no other way of estimating what is the physiological amount of œstrogen circulating in the animal?

FOLLEY: No, I don't know of any. I believe Carl Hartmann has proposed a method of measuring œstrogen on a micro scale in blood, but I don't know that it has ever come into wide use. Perhaps Dr. Gardner could tell us more about it. It seems to me that this method, when we've got more data, might well give us some idea of the physiological output of the rat ovary, provided one can grow the gland faster experimentally than it grows normally. From our experiments, at the moment it looks as though the daily output is between 0 025 and 0 125 micrograms of œstradiol a day, which is rather small.

BEGG: Won't you eventually have to use adrenalectomized ovariectomized animals?

FOLLEY: I suppose we should, but it is very difficult to keep the adrenalectomized animals alive on an œstrogen regime.

BEGG: Have you used salt?

FOLLEY: Dr. Cowie didn't find salt very effective in our rats.

BEGG: How about salt and DCA?

COWIE: Then you would be adding a steroid which might affect the mammary growth.

FOLLEY: DCA, as Dr. Gardner and I showed years ago, does possess mammogenic activity. That was later confirmed by Speert in the monkey.

GARDNER: Did you use any animals, such as the dog, in which œstrogen had no effect on the growth of the mammary gland, or very very slight effect?

FOLLEY: No, I didn't use those.

GARDNER: There is another class, in addition to the two classes you mentioned, in which œstrogens has a very limited effect. Dr. Trentin has recently found that with œstrogen plus progesterone the dogs' mammary glands do very well.

In this analysis of growth of the mammary gland I wondered about the extrapolation with increased doses of œstrogen. We have observed in our laboratory that large doses of œstrogen inhibit mammary growth, disproportionately to total body size. It inhibits general growth too, but if we give crude pituitary extract to keep body growth comparable to normal body growth, œstrogen still inhibits mammary growth. I don't know just where that would fall as far as dosage level is concerned.

I am sure that the dosages you were using here were much below that inhibitory level.

area of all mammary glands in each rat, and, for controls, using other rats. We don't use glands taken before treatment begins from the experimental animals for our controls. Judging by our curves, that

animal.

Boyland: Have you done any experiments with sheep? Sheep should be convenient animals because they are cheaper and more uniform than goats.

Folley. I don't think they're so much cheaper if they are pedigree animals

Sommerville: I think that Dr. Folley would agree that when trying to deduce endogenous physiological hormone production in studies in which the steroid hormones are administered, it would be wrong to neglect the possibility of shifts in the intermediary metabolism which might alter the whole picture. There is evidence for this in pregnancy where there may be an increase in progesterone secretion but, on the other hand, there may be shifts in the intermediary metabolism of the hormone reflected in urinary steroid excretion.

Folley. I think that's an interesting and important point and one that we obviously have to consider. I think that many of these conclusions are rather tentative and subject to considerations of this sort. Would you think that by giving very low physiological doses to spayed animals you may get shifts?

Sommerville: I should think it is less likely.

Folley: In our experiments the doses were of the order of 0 025 μg a day, and such factors should be at a minimum under these conditions.

Hertz Absorption may be a critical factor in your calculations comparing the biological effect of an injected dose and endogenous production.

FOLLEY: I agree that that is possible.

MUHLBOCK: In all your experiments the injections were started at the age of 20 days?

FOLLEY: Yes.

MUHLBOCK: Have you any experience with earlier treatment?

FOLLEY: No, we want to try that in due course.

MUHLBOCK: I have done some experiments to develop the mammary gland in mice in the early days, and there I found no growth. I began the treatment on the first day of life and I continued for 18–20 days.

PULLINGER: I agree with Dr. Muhlbock.

FOLLEY. We suspect that the mammary gland may not be responsive to œstrogen below a certain age. Dr. A. Reynaud of the Institut du Radium in Paris has been working on the effects on the fœtus of steroids administered to the mother, and he believes that the mammary gland is not responsive in late pregnancy. He wrote to us to ask if we had any evidence of what happens during the early period of lactation, from birth to 20 days, and we do intend investigating that.

HERTZ: There must be a marked species difference, because at birth in the human infant there is frequently stimulation.

FOLLEY Yes, I think Dr. Lyon's work on the hormonal basis of "witches milk" is interesting in this connection.

LYONS I think about two-thirds of all new-born infants of both sexes show mammary swelling due to growth of the parenchyma, and not simply to inflammation; and about two-thirds of these secrete milk or colostrum.

BUGG. Isn't the human fœtus biologically more advanced at birth than the rat or the monkey?

LYONS: I think the difference might be in the response of the fœtal mammary glands to the hormones of the placenta.

FOLLEY: Some goats have little udders at birth.

DOBRINER: I would like to ask Dr. Folley whether he has studied other hormones. He mentioned the corticoids. Have you tried the effect of any of the 11-oxygenated steroids on mammary growth?

FOLLEY: Not yet. Unfortunately, we haven't been able to get any cortisone so far. But Dr. Gardner and I showed some years ago that deoxycortone acetate causes mammary growth. We have investigated the effects of adrenalectomy, and concluded that in normal mammary growth the adrenal cortex doesn't seem to be important. In other words, one can by experimental means induce more or less the same degree and rate of mammary growth in adrenalectomized animals as in normal ones.

DOBRINER· The question of DCA is a very tricky one because one of the next intermediates in metabolism is progesterone. We have evidence that it is very easy to reduce the 21-hydroxyl.

LE RÔLE DES HORMONES STEROÏDES DANS LA CROISSANCE NORMALE ET PATHOLOGIQUE DE LA GLANDE MAMMAIRE

A. CHAMORRO

L'OBJET de cette communication ne sera pas, comme le titre pourrait le faire supposer, de présenter une mise au point de l'état actuel de nos connaissances sur les facteurs hormonaux qui déterminent la croissance normale de la glande mammaire ou qui interviennent dans sa pathologie. Tout dernièrement des mises au point ont été publiées par Folley (1947), sur le contrôle endocrinien du développement physiologique de la mamelle, par Nathanson (1947), sur la relation entre les hormones et la pathologie mammaire, et par Lacassagne (1948) sur les hormones et leurs relations avec le cancer et sur la position actuelle du problème de l'action cancérigène des substances œstrogènes (1949). La bibliographie sur les thèmes que nous allons traiter se trouve dans le snotes que, sur la glande mammaire, nous avons présentées depuis 1940 aux Sociétés de Biologie et d'Endocrinologie de Paris.

Nous présenterons une vue d'ensemble des recherches que, sur le rôle des hormones stéroïdes dans le développement normal et pathologique de la glande mammaire, nous avons réalisées au cours de ces dernières années. Nous rapporterons aussi des expériences encore inédites, et les premiers résultats d'autres en cours d'éxécution. Nous ferons, au préalable, une esquisse de l'évolution de nos connaissances sur le déterminisme hormonal de la croissance physiologique de la glande mammaire qui a passé par deux étapes: dans la première, l'ovaire a été considéré comme le centre trophique, dans la deuxième, l'hypophyse détient le rôle le plus important.

La Mamelle sous le Contrôle Hormonal de l'Ovaire

On sait que le développement et l'épanouissement physiologiques de la mamelle sont liés au sexe féminin, mais les données scientifiques sur la subordination de cette glande à la sécrétion interne de l'ovaire ont été seulement apportées au cours du dernier demi-siècle.

Le retentissement que l'extirpation des ovaires a sur la mamelle, a été signalé par Roberts dans son journal de voyage de Delhi à Bombay: des femmes ayant été castrées avant la puberté et examinées par lui à l'âge d'environ 25 ans, présentaient une aplasie de la glande mammaire et du mamelon ainsi qu'une atrophie des organes génitaux. Poth, en 1777, ayant extirpé à une femme les deux ovaires devenus tumoraux, observa que la menstruation cessa et que les seins, très développés, s'affaissèrent. Keppler (1890), ayant réalisé la castration de 46 femmes à cause de maladies gynécologiques, constata une diminution du volume des seins qui ressemblaient à ceux de l'homme, ainsi qu'une diminution de la pigmentation de l'aréole et du mamelon.

La première observation expérimentale de l'influence de l'ovaire sur la mamelle a été rapportée par Hegar en 1878. Cet auteur ayant extirpé un ovaire à une truie et les deux ovaires à une autre, constata que chez la première, les mamelles étaient toujours mieux développées que chez la deuxième. Cette constatation est confirmée par Halban (1900) chez le cobaye et par Foges (1905) chez la lapine impubère chez lesquels la castration empêchait le développement normal des mamelles. Les effets de l'extirpation des ovaires sont complétés par les expériences de greffe réalisées par Knauer, Halban et Steinach. Knauer (1899) réalise la transplantation d'ovaire chez la lapine. Dans le cas de transplantation autoplastique, la greffe prend et les mamelles se développent; dans le cas de transplantation homoplastique, la greffe ne prend pas, l'ovaire est résorbé et les mamelles s'atrophient. Halban (1900) chez le cobaye, constate que la greffe autoplastique d'ovaire provoque un développement

normal de la mammelle. Il admet que l'ovaire produit une substance spécifique nécessaire pour le développement et le maintien de la mamelle et considère l'ovaire comme son centre trophique. Steinach (1912), qui réalise l'implantation d'ovaire chez le cobaye mâle castré, signale que les mamelons, l'aréole et la glande mammaire prennent la forme et le volume de ceux de la femelle normale.

La dépendance de la mamelle à l'ovaire ayant été établie par les observations cliniques et expérimentales, Ancel et Bouin (1909–1911), dans leurs expériences chez la lapine en pseudo-gestation, mettent en lumière le rôle important joué par le corps jaune dans le développement des acini mammaires.

Après la constatation des effets de la castration et de la greffe d'ovaire, la troisième phase expérimentale, l'étude de l'action des extraits glandulaires, est réalisée par Fellner (1913), chez la lapine castrée à l'aide d'extrait ovarien et par Herrmann (1913), chez les lapins mâles ou femelles castrés par administration d'extrait de corps jaune. Ces auteurs constatèrent que ces extraits ovariens stimulent la mamelle. Allen et Doisy (1923) en injectant du liquide folliculaire ou de l'extrait de liquide folliculaire à la souris castrée, ont aussi stimulé la croissance de la glande mammaire.

L'isolement, l'identification chimique et la préparation à l'état pur des deux hormones stéroïdes de l'ovaire, ont permis aux expérimentateurs l'étude de la part qui revient à la folliculine et à la progestérone dans l'édification et les changements physiologiques du réseau mammaire. De nombreuses expériences ont établi que la folliculine développe les tubes mammaires et que l'action synergique de celle-ci et de la progestérone développe les acini. Les études histologiques de la glande mammaire de la femme ont révélé des changements cycliques de l'épithélium mammaire et leur parallélisme avec le cycle sexuel.

La Mamelle sous le Contrôle Hormonal de l'Hypophyse

Les observations de Cushing (1909) et de Aschner (1909)

sur l'atrophie de l'appareil génital consécutive à l'hypophysectomie, et celles de Zondek (1926), de Aschheim (1926) et de Smith (1926) sur l'action stimulante des implantats d'hypophyse sur l'ovaire, ramènent l'hypophyse au rôle dirigeant dans le système endocrinien. L'hypophyse contrôle l'ovaire et, à travers lui, la glande mammaire. Mais ce rôle de l'hypophyse ne se limite pas à l'action exercée par voie indirecte. En 1935, Selye, Collip et Thomson signalaient que l'administration d'œstrone à des rats femelles hypophysectomisés n'empêchait pas l'involution de la mamelle consécutive à l'opération. Lyons et Pencharz (1936) et de nombreux autres expérimentateurs, à leur suite, constatèrent que les œstrogènes étaient incapables de stimuler la mamelle atrophiée par hypophysectomie ou d'entretenir l'hypertrophie provoquée par les œstrogènes avant l'opération.

Les hormones stéroïdes sécrétées par l'ovaire sous l'influence de l'hypophyse ont encore besoin de l'action de l'hypophyse pour être actives sur le récepteur mammaire.

ACTION EXPERIMENTALE DES HORMONES STEROÏDES SUR LA CROISSANCE NORMALE ET PATHOLOGIQUE DE LA GLANDE MAMMAIRE

Croissance Normale

Hormones Stéroïdes de la Gonade

Dans l'édification du réseau mammaire, l'hormone œstrogène était considérée comme responsable de développer seulement les tubes galactophores. Cependant, chez différentes espèces d'animaux (souris, rats, cobayes, lapins et singes castrés), on a obtenu expérimentalement la formation d'acini par la seule administration d'une substance œstrogène.

L'hormone du corps jaune en synergie avec la folliculine était considérée comme chargée de former les acini. Mais, par l'administration de progestérone seule, au rat mâle adulte castré, nous avons provoqué la croissance des tubes et la

formation d'acini. La mamelle, par son extension et sa configuration est aussi comparable à celle de la femelle. Pour obtenir cette action mammogène, il faut cependant une administration journalière de 10–15 mg. d'hormone pendant environ 3 semaines. La souris se montre, dans nos expériences, comme étant beaucoup moins sensible à l'action mammogène de la progestérone. Par contre, chez le cobaye et le lapin castrés, nous n'avons pas obtenu une action stimulante de la mamelle par la seule administration de progestérone même à des doses importantes.

Les androgènes possèdent aussi une action stimulante sur la mamelle, ce qui expliquerait l'existence, chez certaines espèces animales, d'une mamelle relativement développée chez le mâle. Expérimentalement, nous avons constaté que seulement dans les espèces animales où la mamelle du mâle est assez développée, les androgènes (sans fonction œstrogène) ont une action stimulante. Chez la souris, le cobaye et le lapin mâles où la mamelle n'existe qu'à l'état de rudiments, le propionate de testostérone ne montre pas d'activité mammogène. Par contre, chez le rat mâle castré où la mamelle normalement est assez développée, le propionate des testostérone ou la méthyl-testostérone provoquent une stimulation mammaire. Cependant une différence apparait entre les rats mâles et femelles adultes, castrés. Chez les premiers, l'androgène agit sur les tubes sans provoquer la formation d'acini; chez les deuxièmes, le propionate de testostérone agit sur les tubes et développe les acini.

Bien que la progestérone et le propionate de testostérone stimulent la glande mammaire, leur action est fondamentalement différente. La progestérone féminise la mamelle. Agissant sur une mamelle de mâle atrophiée par castration, elle stimule les tubes, provoque l'apparition de bourgeons de croissance à l'extrêmité périphérique du réseau mammaire qui dépasse ses limites primitives, et développe des acini. Le propionate de testostérone ne féminise pas la mamelle: il stimule les tubes pré-formés, provoque leur ouverture et même leur élargissement, mais il est incapable de provoquer

la néo-formation de tubes par apparation de bourgeons de croissance ni la néo-formation d'acini mammaires; chez la femelle où les acini existent, il les développe. Il n'agit, en somme, que sur les parties de l'organe pré-existant à l'état d'ébauche, en provoquant leur hypertrophie et leur dilatation.

Hormones Stéroïdes Cortino-mimétiques

La désoxycorticostérone a été considérée, par différents auteurs, comme une substance capable de stimuler la glande mammaire, mais dans les expériences que nous avons réalisées chez la souris et le rat, ce pouvoir se montre très faible et inconstant malgré les fortes doses administrées. Parfois, chez les rats, un animal isolé présente une légère stimulation. Comme pour la progestérone et les androgènes, il existe une différence de sensibilité selon l'espèce animale envisagée. Chez le cobaye femelle castré, nous avons obtenu un développement mammaire par l'administration d'acétate de désoxycorticostérone.

La synergie qu'on obtient avec l'association d'un œstrogène et de la progestérone peut être aussi constatée en remplaçant cette dernière par l'acétate de désoxycorticostérone. Nous avons observé cette action chez le cobaye et le lapin castrés.

L'expérimentation a détruit la conception classique selon laquelle les œstrogènes provoquaient exclusivement la formation des tubes galactophores, et l'association d'œstrogène et de progestérone l'apparition d'acini. Cependant, pour obtenir, par une seule hormone le développement complet de la mamelle, il faut employer des quantités non physiologiques, parfois massives, de ces hormones. C'est avec l'association synergique de faibles doses d'œstrogène et de progestérone qu'on obtient le plus facilement le développement physiologique de la glande mammaire.

Antagonisme des Hormones Stéroïdes sur la Croissance Mammaire

Le fait que les substances œstrogènes et androgènes s'opposent dans leur action sur le vagin, a suggéré la recherche

d'un antagonisme des hormones stéroïdes sur le récepteur mammaire. Il faut cependant remarquer que cet antagonisme ne consiste pas dans une inhibition de l'action de l'œstrogène, mais dans une transformation de son action qui, d'œstrogène, se mue en progestative, l'action kératinisante se transformant en une action mucifiante. D'autre part, en raison du mécanisme spécial d'action des stéroïdes sur le récepteur mammaire, celui-ci ne peut être assimilé à d'autres récepteurs comme le vagin, la muqueuse utérine ou la prostate. Sur ceux-ci, toute action des hormones stéroïdes agissant seules ou associées (stimulation, synergie ou antagonisme) se réalise directement et peut être mise en évidence chez les animaux privés d'hypophyse. Par contre, pour toute action des hormones stéroïdes sur la mamelle, il faut le concours de l'hypophyse. Le problème de l'antagonisme des hormones sexuelles sur la glande mammaire doit être envisagé en tenant compte de cette nouvelle acquisition que toute action des hormones stéroïdes dépend, en définitive, de l'hypophyse.

Pour la glande mammaire, de nombreux essais expérimentaux et thérapeutiques ont été réalisés pour mettre à profit cette action antagoniste de l'hormone mâle et femelle: prophylaxie et traitement de l'adéno-carcinome mammaire spontané ou provoqué par l'œstrogène, chez la souris; traitement de l'hyperplasie kystique et du cancer mammaires chez la femme; et prophylaxie de récidives de ce dernier.

Pour tâcher d'éclaircir le mécanisme de l'action d'inhibition des androgènes, recherché sur la cellule mammaire cancérisée, il était intéressant de préciser s'il serait possible d'inhiber, par un androgène, la croissance normale de l'épithélium mammaire provoquée par un œstrogène. Pour ceci, de fortes doses d'une substance androgène ont été opposées à de faibles quantités d'œstrogène, suffisantes cependant pour provoquer un développement mammaire.* Nous avons choisi comme animaux d'expérience la souris, le cobaye et le lapin mâles, chez lesquels, au préalable, nous avions constaté que de

*Expériences inédites.

fortes doses d'androgène restaient sans action sur les ébau
mammaires des animaux castrés.

Expériences chez la souris: Un groupe de souris mâles l
a été traité avec 10 μg. de benzoate d'œstradiol, admin
deux fois par semaine, par la voie sous-cutanée. Un deux
groupe, en plus de l'œstrogène a reçu simultanément une
200 fois supérieure de méthyl-testostérone. Toutes
mamelles des animaux ont été étudiées *in toto*, à trois, si
huit semaines d'intervalle. Jusqu'à trois semaines, on cons
une inhibition partielle de la croissance, l'aire mamm
est plus réduite et les bourgeons de croissance moins no
breaux, mais dans les délais de six et huit semaines, le trai
ment associé s'est transformé en une action synergiq
semblable à celle qui s'obtient avec la progestérone; l'a
mammaire est plus étendue, les acini sont beaucoup pl
nombreux, la dilatation des tubes est plus modérée.

Expériences chez le cobaye: Chez le cobaye, on administ
soit 20 μg. de benzoate d'œstradiol, soit la même quanti
d'œstrogène associé à une dose 200 ou 800 fois supérieure d
propionate de testosterone, trois fois par semaine. Les
mamelles sont étudiées aprés trois, six, neuf et douze semaine
de traitement. Au terme de trois semaines, on constate
comme chez la souris, une inhibition de la croissance de
tubes qui sont aussi plus dégarnis. A partir de six semaines
on ne remarque plus de différences dans l'aire mammaire des
deux groupes et les mamelles, très hypertrophiées, présentent
des tubes et des acini remplis de sécrétion.

Expériences chez le lapin: Chez le lapin, nous avons opposé
à la dose d'œstrogène, une autre mille fois supérieure de
propionate de testostérone: 5 μg. de benzoate d'œstradiol et
la même dose associée à 5 mg. de propionate de testostérone,
sont adminitrés, six fois par semaine. La deuxième paire de
mamelles des animaux du groupe recevant seulement l'œstrogène et du groupe à traitement associé, a été étudiée *in toto*
après deux, trois et quatre semaines de traitement. Au bout
de deux semaines, les mamelles du deuxième groupe, l'aire
étant similaire, présentent un réseau de tubes plus serrés et

moins dilatés. A la troisième semaine, on constate une aire mammaire inférieure dans la plupart des animaux, mais le réseau de tubes est toujours plus serré avec des bourgeons de croissance plus nombreux. A la quatrième semaine, l'aire mammaire est parfois inférieure, d'autres fois égale ou supérieure à celle des animaux traités par l'œstrogène seul. Chez les animaux où l'action synergique apparait, celle-ci se traduit ou par l'augmentation de l'aire mammaire, ou par la qualité de la réaction qui ressemble à celle qu'on obtient avec un traitement associé d'œstrogène et de progestérone ou par l'association de ces deux facteurs (Fig. 1).

L'action d'inhibition de croissance constatée à la troisième semaine, chez les trois espèces animales, se transforme en action synergique.

Avec ces mêmes doses d'œstrogène et de progestérone, nous n'avons pas pu mettre en évidence l'action antagoniste sur la mamelle du lapin qui a été observée par Lyons et MacGinty (Fig. 2).

L'expérimentation ne permet pas de conclure à un véritable antagonisme entre œstrogènes et androgènes. L'association de ceux ci fait apparaître que l'androgène agit à la façon de la progestérone.

Inhibition de la Croissance Mammaire par les Œstrogènes

Les substances œstrogènes administrées d'une façon réitérée, ont la propriété d'inhiber la sécrétion de certaines hormones hypophysaires: somatotrope, gonadotropes et thyréotrope. Etant donné que les œstrogènes agissent sur la glande mammaire avec la coopération d'un facteur hypophysaire, on conçoit la possibilité d'agir sur l'hypophyse pour essayer d'inhiber, par de fortes doses d'œstrogène, la sécrétion de cette substance hypophysaire mammogène. Gardner a signalé que 50 μg. d'œstrogène, par semaine, avaient un pouvoir inférieur pour stimuler la mamelle de la souris que des doses plus faibles. Nous avons confirmé cette observation chez les rats mâles castrés, chez lesquels 25 μg. par jour d'une

substance œstrogène provoquent un développement supérieur à celui obtenu avec 250 µg. par jour.

L'action thérapeutique de fortes doses d'œstrogène, que Haddow et ses collaborateurs ont observée sur le cancer mammaire de la femme, pourrait s'expliquer par cette action indirecte *par voie hypophysaire*.

Croissance Pathologique

Expérimentalement, l'administration réitérée d'une substance œstrogène aboutit à la formation d'une hyperplasie kystique de la mamelle. Cependant, toute hormone stéroïde, capable de stimuler la mamelle, ne produit pas cette action pathologique. Nous avons constaté que l'éthinyl-testostérone *ou pregneninolone, stéroïde synthétique, ayant des propriétés* œstrogène, progestative et androgène, est capable rapidement de féminiser la mamelle de la souris mâle, mais cependant, n'aboutit pas à provoquer la dégénérescence kystique. La progestérone, administrée d'une façon réitérée chez les rats où elle est active sur la mamelle, ne provoque pas non plus d'hyperplasie kystique. Les androgènes, par contre, administrés chez les rats mâles castrés, aboutissent, aprés un traite*ment réitéré de plusieurs semaines à provoquer* des tubes dilatés d'aspect kystique.

Depuis que Goomaghtigh et Amerlinck (1930), par l'administration d'extrait de liquide folliculaire, ont produit des formations adénomateuses et l'hyperplasie kystique de la femme. Mazer (1934), pensant à l'antagonisme hormonal, a suggéré de traiter cette maladie par les androgènes, et à la suite des résultats cliniques rapportés par Desmaret et Capitain (1937) *cette thérapeutique de la maladie de Reclus* est couramment employée.

Nous avons essayé de voir si cette thérapeutique pouvait être basée sur des données expérimentales. Pour cela, des rats femelles castrés ont reçu une quantité d'œstrogène

suffisante pour provoquer une hyperplasie kystique et à d'autres, nous avons associé à ce traitement le propionate de testostérone.* Les animaux ont reçu, soit 10 µg. de benzoate d'œstradiol par jour, pendant 4–6 semaines, soit la même dose d'œstrogène associée à une dose de propionate de testostérone 100 et 200 fois supérieure. L'étude des mamelles *in toto* et en coupes montre qu'au lieu d'empêcher l'apparition de l'hyperplasie kystique, le propionate de testostérone agit d'une façon synergique en provoquant des hypertrophies et hyperplasies plus importantes. Il semblerait donc que, si le propionate de testostérone agit d'une façon salutaire sur la mastopathie kystique, ce soit par un mécanisme autre que celui de l'antagonisme entre œstrogènes et androgènes.

RÔLE DE L'HYPOPHYSE DANS L'ACTION DES HORMONES STEROÏDES SUR LA GLANDE MAMMAIRE

Action des Hormones Stéroïdes Seules ou Associées en Absence de l'Hypophyse

Depuis la première constatation négative de Selye, Collip et Thomson, de nombreuses recherches ont été réalisées pour tâcher de stimuler la glande mammaire des animaux hypophysoprivés, par l'administration isolée ou associée d'hormones stéroïdes. La plupart des auteurs, dans des expériences réalisées chez la souris, le rat et le cobaye, ont constaté des résultats négatifs. Cependant, dans ces dernières années des travaux ont encore attribué aux œstrogènes ou aux androgènes une action stimulante sur la mamelle du rat hypophysectomisé. Nous avons réalisé des expériences successives afin d'étudier l'action que les hormones stéroïdes seules ou associées, l'extrait d'ovaire et la sécrétion du propre ovaire de l'animal pourraient avoir sur la mamelle du rat hypophysectomisé.

*Expérience inédite.

Nous avons signalé qu'une association d'œstrogène et d'acétate de désoxycorticostérone provoque une stimulation mammaire chez le rat mâle hypophysectomisé. Cette expérience a été confirmée par Gardner chez la souris hypophysectomisée. Mais cette stimulation est relativement faible et il faut la ramener à sa vraie place. Parmi les nombreuses expériences que nous avons réalisées, nous en avons retenu certaines qui, à cause du long traitement et des hautes doses administrées, permettront de se rendre compte de l'importance toute relative de cette stimulation. Les détails sont consignés dans le tableau ci-joint Table I.

De l'analyse de ces cas, il résulte qu'une stimulation peut être obtenue chez le rat hypophysectomisé par l'administration d'un œstrogène naturel ou par l'association de cet œstrogène et de progestérone ou d'acétate de désoxycorticostérone. Mais, cette stimulation reste limitée au territoire *des gros tubes collecteurs aboutissant au mamelon* et est très faible, étant données et la durée du traitement, et les doses massives administrées. Or, avec des doses très inférieures, chez l'animal castré, on provoque d'énormes hyperplasies *et même des phénomènes pathologiques.* Il semble que l'épithélium mammaire des gros tubes collecteurs aboutissant au mamelon, soit un épithélium de transition jouissant, en un certain degré, de la propriété de réagir comme le *mamelon réagit chez les animaux sans hypophyse.* Nous n'avons, par contre, jamais constaté de stimulation du réseau mammaire secondaire situé au delà de ces gros tubes collecteurs. Avec de très fortes doses d'un œstrogène artificiel, *nous n'avons même pas observé cette action stimulante* limitée à la zone proche du mamelon. Il semble qu' une différence se manifeste entre œstrogènes naturels et artificiels dans ce cas particulier.

Action des Extraits Ovariens en Absence de l'Hypophyse

Il nous a semblé intéressant de vérifier si un extrait d'ovaire n'avait pas, pour stimuler la mamelle, de capacités différentes

TABLE I

ACTION D'UNE SUBSTANCE ŒSTROGÈNE SEULE OU ASSOCIÉE A LA PROGESTÉRONE OU A L'ACÉTATE DE DÉSOXYCORTICOSTÉRONE SUR LA MAMELLE DU RAT HYPOPHYSECTOMISÉ

Sexe	Survie de l'animal après hypophysectomie (en jours)	Durée du traitement (en jours)	Voie d'administration	Dose totale d'hormone stéroïde administrée en mg.			Réaction mammaire	Poids initial en g.	Poids final en g.	Température C° rectale profonde le jour du sacrifice
				Œstrogène	Progestérone	Acétate de désoxycorticostérone				
femelle castrée	87	0	-	0	0	0	Atrophie	151	125	35°9
femelle castrée	87	80	comprimé de 10,0 mg inséré sous la peau	D.S.B.(1) 8,5			Atrophie	143	98	35°8
mâle	81	0	—	0	0	0	Atrophie	158	132	36°8
mâle	81	50	voie orale	D.S.B. 11,45	0	0	Atrophie	174	105	32°8
femelle castrée	98	0	—	0	0	0	Atrophie	118	85	36°
femelle castrée	98	92	comprimé de 8 mg inséré sous la peau	B.E.(2) 1,6	0	0	+	140	93	non prise
mâle	70	0	—	0	0	0	Atrophie	149	108	36°5
mâle	70	50	sous-cutanée	B.O.(3) 1,6	51	0	Atrophie	148	103	35°
mâle	98	0	—	0	0	0	Atrophie	120	95	34°8
mâle	98	84	sous-cutanée	B.O. 2,4	75	0	±	151	95	31°2
femelle	84	0	—	0	0	0	Atrophie	64	86	35°5
femelle	84	50	sous-cutanée	B.O. 3,1	0	8	±	50	55	31°
mâle	98	0	—	0	0	0	Atrophie	121	124	36°7
mâle	98	50	sous-cutanée	B.O. 2,2	0	11,5	+ (4)	188	92	non prise

(1) D.S.B. = Diéthylstilbœstrol, (2) B.E. = Benzoate d'œstrone, (3) B.O. = Benzoate d'œstradiol.
(4) Légère stimulation.

Nous avons signalé qu'une association d'œstrogène et d'acétate de désoxycorticostérone provoque une stimulation mammaire chez le rat mâle hypophysectomisé. Cette expérience a été confirmée par Gardner chez la souris hypophysectomisée. Mais cette stimulation est relativement faible et il faut la ramener à sa vraie place. Parmi les nombreuses expériences que nous avons réalisées, nous en avons retenu certaines qui, à cause du long traitement et des hautes doses administrées, permettront de se rendre compte de l'importance toute relative de cette stimulation. Les détails sont consignés dans le tableau ci-joint Table I.

De l'analyse de ces cas, il résulte qu'une stimulation peut *être obtenue chez le rat hypophysectomisé par l'administration* d'un œstrogène naturel ou par l'association de cet œstrogène et de progestérone ou d'acétate de désoxycorticostérone. Mais, cette stimulation reste limitée au territoire des gros tubes collecteurs aboutissant au mamelon et est très faible, étant données et la durée du traitement, et les doses massives administrées. Or, avec des doses très inférieures, chez l'animal castré, on provoque d'énormes hyperplasies et même des phénomènes pathologiques. Il semble que l'épithélium mammaire des gros tubes collecteurs aboutissant au mamelon, soit un épithélium de transition jouissant, en un certain degré, de la propriété de réagir comme le mamelon réagit chez les animaux sans hypophyse. Nous n'avons, par contre, jamais constaté de stimulation du réseau mammaire secondaire situé au delà de ces gros tubes collecteurs. Avec de très fortes doses d'un œstrogène artificiel, nous n'avons même pas observé cette action stimulante limitée à la zone proche du mamelon. Il semble qu' une différence se manifeste entre œstrogènes naturels et artificiels dans ce cas particulier.

Action des Extraits Ovariens en Absence de

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Sexe	Survie de l'animal après hypophysectomie (en jours)	Durée du traitement (en jours)	Voie d'administration	Dose totale d'hormone stéroïde administrée en mg.			Réaction mammaire	Poids initial en g	Poids final en g	Température C° rectale profonde le jour du sacrifice
				Œstrogène	Progestérone	Acétate de désoxycorticostérone				
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femelle castrée	87	80	comprimé de 10,6 mg inséré sous la peau	D S.B.(1) 8,5			Atrophie	143	98	35°8
mâle	81	0	—	0	0	0	Atrophie	158	132	36°8
mâle	81	56	voie orale	D.S.B. 11,45	0	0	Atrophie	174	105	32°8
femelle castrée	98	0	—	0	0	0	Atrophie	113	85	36°
femelle castrée	98	92	comprimé de 8 mg inséré sous la peau	B.E (2) 1,6	0	0	±	140	93	non prise
mâle	70	0	—	0	0	0	Atrophie	148	108	36°5
mâle	70	56	sous-cutanée	B.O.(3) 1,6	51	0	Atrophie	148	103	35°
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mâle	98	84	sous-cutanée	B.O 2,4	75	0	±	151	95	31°2
femelle	84	0	—	0	0	0	Atrophie	64	66	35°5
femelle	84	56	sous-cutanée	B.O. 3,1	0	8	±	50	55	31°
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(1) D.S B. = *Diéthyl-Stilbœstrol*, (2) B E. = *Benzoate d'œstrone*, (3) B O = *Benzoate d'œstradiol*.
(±) Légère stimulation.

à celles des hormones stéroïdes cristallisées. Cette expérience, qui a été réalisée en collaboration avec Girard et Sandulesco, est restée à l'état de premier résultat,* et une étude approfondie avec des extraits concentrés et fractionnés n'a pas été réalisée. Toutefois, un extrait brut d'ovaire de truie a été administré, chez le rat mâle hypophysectomisé, à mamelles atrophiées, et nous avons constaté une faible stimulation mammaire qui, à l'inverse de celle qui a été obtenue auparavant avec les hormones synthétiques, se localise dans les *tubes du réseau périphérique*. *Etant donné qu'il s'agit d'un extrait brut, la dose des substances actives est certainement* faible, et sans aucun rapport avec les quantités très importantes d'hormones stéroïdes cristallisées que nous avons administrées dans l'expérience précédente.

Action des Hormones Stéroïdes Secretées par l'Ovaire, sous l'Influence de la Gonadotrophine Sérique de Jument Gravide, sur la Mamelle des Animaux sans Hypophyse

Les substances œstrogènes, même à des doses peu élevées, administrées journellement, provoquent l'amaigrissement des animaux normaux. Mais leur action toxique devient considérable si elles sont administrées à des animaux sans hypophyse. Ces animaux supportent mal le traitement et même quand on réussit à les maintenir en équilibre, il en résulte une chute de poids plus considérable que chez les témoins, une perte plus importante de l'appétit et une baisse plus accentuée de la température rectale profonde. Cette baisse de température qui, chez les animaux hypophysectomisés témoins atteint 2–3 degrés, se chiffre de 5–7 degrés chez les animaux traités par des substances œstrogènes. Dans les expériences réalisées avec des extraits bruts d'ovaire, la solution huileuse, administrée en quantités importantes, se résorbe mal, bien que les animaux se maintiennent dans un état général plus satisfaisant.

*Expérience inédite.

Nous avons constaté par contre que l'administration de gonadotrophine, chez les animaux sans hypophyse, n'était suivie ni d'amaigrissement, ni de chute de température plus considérable. Nous avons donc envisagé de stimuler l'ovaire par une gonadotrophine, pour voir la répercussion que ce traitement aurait sur la mamelle des animaux sans hypophyse. Nous avons choisi la gonadotrophine du serum de jument gravide, active sur les ovaires des animaux sans hypophyse, et d'origine non hypophysaire, puisque sécrétée par le placenta. D'autre part, la propre sécrétion de l'ovaire de l'animal allait agir sur la mamelle. Pour ceci, de jeunes rats femelles, vierges, d'un poids de 100 à 150 g. ont été hypophysectomisés. Environ trois jours aprés l'opération, avant que l'ovaire et la mamelle n'entrent en régression, on commence le traitement par voie sous-cutanée ou intra-péritonéale. matin et soir, à la dose journalière de 20 à 30 unités-rat, pendant une période de 3–4 semaines. Les ovaires des animaux traités atteignent des poids considérables de 200 à 483 mg. Ils sont remplis de gros follicules parfois hémorragiques et de nombreux corps jaunes. Les ovaires des témoins pèsent de 10 à 15 mg. L'étude comparative, *in toto* et en coupes, des mamelles de ces deux groupes d'animaux, montre, que chez les animaux traités, l'involution mammaire est empêchée (Fig. 3). Si un tel traitement est poursuivi pendant plusieurs semaines, l'ovaire devient réfractaire à la stimulation, entre en involution et la mamelle régresse.

Ce résultat pourrait être attribué à une action synergique de la gonadotrophine et de la sécrétion ovarienne. Or, chez les rats castrés et hypophysectomisés, l'administration de gonadotrophine associée à un stéroide œstrogène et progestatif n'a pas empêche l'involution mammaire. Il faudrait interpréter cette constatation en admettant, ou bien que la sécrétion du propre ovaire de l'animal se montre plus actif sur la mamelle, ou bien que le bon état général de celui-ci favorise la réaction mammaire.

Action, sur la Glande Mammaire, des Implantats d'Hypophyses

La constatation que les substances œstrogènes sont inactives [illegible] Turner et ses collabora- [illegible] 'existence d'hormones [illegible] et qui stimuleraient directement la mamelle. Une hormone mammogène serait sécrétée sous l'influence de la folliculine et provoquerait la croissance des tubes, une deuxième serait sécrétée sous l'influence de la progestérone et produirait la prolifération d'acini, la folliculine et la progestérone n'ayant d'autre rôle que d'exciter l'hypophyse. Cette hypothèse prenait assise sur la constatation expérimentale que les hypophyses des animaux soumis à un traitement œstrogène et celles des animaux gravides étaient seules capables de stimuler la mamelle des animaux castrés-hypophysectomisés.

Nous avons réalisé des expériences pour déceler l'existence de ces hormones hypothétiques.

A des rats mâles hypophysectomisés, à mamelles atrophiées, nous avons fait des implantations répétées de trois sortes d'hypophyses provenant de rats normaux, de rats castrés ou de rats ayant subi au préalable un traitement œstrogène pendant trois mois. Les deux premières sortes d'hypophyses provoquent une stimulation mammaire. Par contre, les hypophyses de la troisième catégorie ne produisent qu'une très faible stimulation. Dans les deux premiers cas, les hypophyses implantées ont stimulé les testicules, dans le troisième, les hypophyses, pauvres en hormones gonadotropes, les ont à peine stimulé. Si la mamelle, dans ce dernier cas, n'a pas été stimulée, il semble que ce soit à cause du manque de sécrétion d'hormones stéroïdes par la gonade, d'où manque d'un des éléments nécessaires pour l'action synergique. Pour vérifier cette interprétation, chez les rats castrés-hypophysectomisés, des hypophyses provenant d'animaux œstrogénisés sont implantées en leur associant l'administration d'une hormone stéroïde mammogène. Dans ce cas, on



A



B

FIG 3 A Mamelle atrophiée de jeune rat femelle vierge, 6 semaines après l'hypophysectomie B idem, après administration pendant les 5 dernières semaines de gonadotrophine sérique

Action, sur la Glande Mammaire, des Implantats d'Hypophyses

La constatation que les substances œstrogènes sont inactives en l'absence de l'*hypophyse, a conduit Turner et ses collaborateurs*, à émettre l'hypothèse de l'existence d'hormones spécifiques sécrétées par l'hypophyse et qui stimuleraient directement la mamelle. Une *hormone mammogène* serait sécrétée sous l'influence de la folliculine et provoquerait la croissance des tubes, une deuxième serait sécrétée sous l'influence de la progestérone et produirait la prolifération d'acini, la folliculine et la progestérone n'ayant d'autre rôle que *d'exciter l'hypophyse. Cette hypothèse prenait* assise sur la constatation expérimentale que les hypophyses des animaux soumis à un traitement œstrogène et celles des animaux *gravides étaient seules capables de stimuler* la mamelle des animaux castrés-hypophysectomisés.

Nous avons réalisé des expériences pour déceler l'existence de ces hormones hypothétiques.

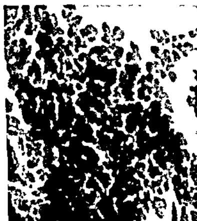
A des rats mâles hypophysectomisés, à mamelles atrophiées, nous avons fait des *implantations répétées* de trois sortes d'hypophyses provenant de rats normaux, de rats castrés ou de rats ayant subi au préalable un traitement œstrogène pendant trois mois. Les deux premières sortes d'hypophyses provoquent une stimulation mammaire. Par contre, les *hypophyses de la troisième catégorie ne produisent* qu'une très faible stimulation. Dans les deux premiers cas, les hypophyses implantées ont stimulé les testicules, dans le troisième, les hypophyses, pauvres en hormones gonadotropes, les ont à peine stimulé. Si la mamelle, dans ce dernier cas, n'a pas *été stimulée*, il semble que ce soit à cause du manque de sécrétion d'hormones stéroïdes par la gonade, d'où manque d'un des éléments nécessaires pour l'action synergique. *Pour* vérifier cette interprétation, chez les rats castrés-hypophysectomisés, des hypophyses provenant d'animaux œstrogénisés sont implantées en leur associant l'administration d'une hormone stéroïde mammogène. Dans ce cas, on



A



B



C



D

Fig 5 A Mamelle de rat femelle adulte normal Témoin
B Ide
Stimul
traiten
nombr
dilatés





A B



C

FIG. 4. A Mamelle de jeune rat mâle castré, 110 hours après

's
·)
-
x

bourgeons de croissance. ×6,5

onstate une très nette stimulation mammaire (Fig. 4). Cette xpérience montre que l'hormone stéroïde est nécessaire pour btenir une stimulation mammaire plus importante.

Cependant les hypophyses des rats normaux possèdent la apacité de stimuler la mamelle du rat hypophysectomisé et astré. Mais dans ce cas, la stimulation est faible et se aractérise par l'apparition de petits bourgeons de croissance. 'action devient plus intense si, en plus des hypophyses, on dministre une hormone stéroïde mammogène. L'interven-ion dans la réaction d'autres glandes endocrines n'est pas écarter puisque si, à des rats castrés, à mamelle atrophiée, n extirpe la thyroïde et la surrénale, et on réalise des im-lantations d'hypophyses, la stimulation mammaire est nulle u très faible. D'autre part Lacassagne et Dubois n'ont as observé un pouvoir particulier des hypophyses des souris ravides pour stimuler la mamelle de la souris castrée.

Si l'hypophyse secrète une hormone spécifique pour stimuler irectement la glande mammaire, cette hormone doit se :ouver dans le sang des animaux à glandes mammaires forte-ent développées. Nous avons essayé de la déceler dans le :rum sanguin des animaux soumis à un traitement réitéré ar les œstrogènes et dans le serum de jument gravide et de mme enceinte. Ces produits administrés à des rats castrés-ypophysectomisés n'ont pas donné de stimulation mammaire.

Ces expériences montrent que l'hypophyse et les hormones

ussi contredite par les constatations de Mussio-Fournier, lbrieux et Buño (1937) et Lyons et Sako (1940) sur l'activité es œstrogènes en application locale.

Synergie des fractions hypophysaires et des hormones stéroïdes ammogènes. La synergie des différentes fractions hypo-hysaires avec les œstrogènes a été recherchée en utilisant le t castré-hypophysectomisé. Nous avons essayé les fractions onadotrope, thyréotrope et la prolactine, et seulement cette ernière associée à l'œstrogène stimulait la mamelle. Ce



A



B

Fig 6 A Mamelle de rat femelle adulte Témoin B Idem, après administration de propyl-thiouracile (un mg par jour) pendant 6 semaines Dilatation des tubes qui contiennent une sécrétion, et prolifération d'acini. ×44

d'hypothyroïdisme crée un terrain favorable à une action intensifiée des hormones stéroïdes qui conduit à l'apparition des lésions pathologiques mammaires.

Dans une série d'expériences, nous avons mis en évidence le rôle de la thyroïde dans le déclanchement des phénomènes pathologiques mammaires par les hormones stéroïdes.

(i) L'administration à des rats castrés-thyroïdectomisés d'un stéroïde mammogène provoque une action plus intense que chez les rats seulement castrés.

(ii) Des rats femelles adultes sont thyroïdectomisés et traités par une gonadotrophine à la dose de 10 u.i. par jour, pendant quelques jours. Tandis que chez les animaux normaux traités, on constate une légère stimulation mammaire, chez les thyroïdectomisés, les mamelles sont le siège d'une hypertrophie et d'une hyperplasie qui prennent parfois un caractère kystique (Fig. 5).

(iii) Nous avons ensuite étudié la répercussion mammaire de la production chez les rats femelles adultes, âgés d'environ 14 mois, d'un état d'hypothyroïdisme léger. Les animaux ont été soumis à l'administration sous-cutanée de faibles doses de propyl-thiouracile et les mamelles étudiées *in toto* et en coupes à des délais variant de 4 à 10 semaines. Nous avons constaté dans les premières semaines une prolifération d'acini et ensuite l'apparition de phénomènes d'hyperplasie kystique. Parfois, il s'agit seulement d'une extrême dilatation des tubes; d'autres fois, de prolifération de grappes d'acini devenus kystiques, parsemés dans la glande; d'autres fois, d'apparition de grands kystes isolés (Fig. 6). Ces manifestations rapellent les différents degrés de la mastopathie kystique chez la femme.

(iv) Dans une autre expérience, nous avons étudié quel serait l'effet sur la glande mammaire, de l'ablation de la thyroïde* chez le rat femelle adulte, âgé de 14 à 18 mois. L'examen des mamelles est réalisé à des délais de 4 à 10

*Pendant les 10 jours précédant la thyroïdectomie, les animaux reçoivent 1 mg de propyl-thiouracile par jour, par la voie sous-cutanée ou intrapéritonéale, ce qui réduit pratiquement à zéro la mortalité consécutive à l'opération.

résultat a été aussi rapporté par différents auteurs avec les fractions de croissance et corticotrope.

Toutefois, ces expériences ont seulement une valeur d'orientation et l'existence d'une hormone mammogène ne pourra être prouvée que lorsqu'on disposera d'hormones hypophysaires à l'état pur.

Mécanisme de l'Action Stimulante des Hormones Stéroïdes sur la Glande Mammaire

Le mécanisme d'action des hormones stéroïdes sur la croissance normale de la glande mammaire se présente, à l'heure actuelle, comme un problème complexe très loin de la simplicité de la conception première d'un déterminisme ovarien. Les substances œstrogènes ne sont actives sur la glande mammaire qu'avec la coopération hypophysaire. Mais le mécanisme de cette coopération n'est pas éclairci. Cette action associée peut s'expliquer par:

(*a*) une action préparatoire ou mordançage, exercée par l'hypophyse sur l'épithélium mammaire.

(*b*) une synergie entre une substance hypophysaire et l'hormone stéroïde.

(*c*) une transformation de la constitution chimique de la substance stéroide produite sous l'influence de l'hypophyse et rendant celle-là active sur l'épithélium mammaire.

Le métabolisme de l'hormone stéroïde serait provoqué *in situ* par des enzymes contrôlés par l'hypophyse. Chez les animaux hypophysectomisés, ces enzymes disparaitraient ou deviendraient inactifs, d'où manque d'activité de l'œstrogène introduit dans la circulation générale ou appliqué *in situ*. Cette hypothèse permettrait un accord entre le rôle joué par l'hypophyse et l'action locale exercée par les œstrogènes.

INTERVENTION DE LA THYROÏDE DANS L'ACTION DES HORMONES STEROÏDES SUR LA GLANDE MAMMAIRE

Dans la coopération de l'ovaire et de l'hypophyse pour agir sur la croissance mammaire, la thyroïde intervient. Un état



A



B



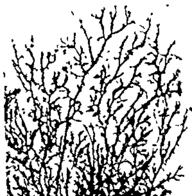
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semaines après l'opération et nous avons constaté, chez la plupart des animaux, une dilatation des tubes, une prolifération d'acini, des formation adénomateuses et l'apparition d'acini kystiques, soit en grappes isolées, soit généralisés à toute la glande. L'administration de thyroxine empêche la réaction mammaire (Fig. 7).

(v) Chez le rat femelle castré, l'administration d'une quantité de benzoate d'œstradiol de 2·5 μg. trois fois par semaine, capable, à peine, d'empêcher l'involution de la mamelle, produit chez le rat castré-thyroïdectomisé, la dilatation des tubes et une abondante prolifération d'acini en grande partie kystiques. Si les rats castrés sont soumis à un traitement par le benzoate d'œstradiol à une dose de 5 μg., trois fois par semaine, on obtient, au bout de six semaines de traitement, des manifestations très modérées d'hyperplasie kystiques; si à ces mêmes animaux, on ajoute un traitement par la thyroxine, on empêche l'apparition de toute stimulation normale ou pathologique (Fig. 8).

(vi) Dans cette expérience, nous avons étudié l'action que le propionate de testostérone pourrait avoir sur la mamelle dans un état d'hypothyroïdisme (expérience inédite). Des rats femelles adultes âgés de 14 à 18 mois sont castrés et traités, soit par le propionate de testostérone, soit par une association de propyl-thiouracile et de propionate de testostérone, soit thyroïdectomisés et soumis au traitement par le propionate de testostérone. Le propyl-thiouracile et l'androgène ont été administrés aux doses journalières d'un ou deux milligrammes.

Les femelles castrées traitées par le propionate de testostérone, présentent une stimulation mammaire qui dépasse l'état normal, avec élargissement des tubes. Chez les animaux des deux autres groupes, propyl-thiouracile ou thyroïdectomie associés à l'androgène, la mamelle est hypertrophiée et hyperplasiée avec signes de dégénérescence kystique (Fig. 9). Microscopiquement, on observe une dilatation des tubes et des acini qui contiennent une sécrétion; des proliférations épithéliales de type papilliforme et une desquamation épithéliale



A



B



C



D

FIG 9 A Mamelle de rat femelle adulte, castré depuis 6 semaines, atrophie B Idem, castré, traité par le propionate de



A



B



C



D

FIG 8 A. Mamelle de rat femelle adulte castré, après administration de 2 5 µg de benzoate d'œstradiol, 3 fois par semaine, pendant 6 semaines B Idem, castré et thyroïdectomisé et recevant le même traitement que le precedent, hyperplasie kystique

abondante contenant des cellules spongieuses à l'intérieur des tubes et des acini, ainsi qu'une réaction conjonctive périglandulaire, parfois intense, toutes ces lésions étant semblables à celles qu'on observe dans la maladie kystique, chez la femme (Fig. 10). La muqueuse utérine de ces animaux présente aussi une hyperplasie kystique.

D'après ces expériences, dans les états d'hypothyroïdisme, les androgènes peuvent agir d'une façon pathologique sur la glande mammaire. Il faudrait rapprocher ces résultats du fait que, dans la clinique, la mastopathie et le cancer mammaire apparaissent le plus souvent à un âge où la fonction thyroïdienne est à son déclin et où la sécrétion androgène de l'ovaire augmente.

Le mécanisme de production expérimentale de l'hyperplasie kystique par les œstrogènes serait le suivant: les substances œstrogènes agissent sur l'hypophyse en y provoquant une diminution et, à la longue, une suspension de la sécrétion d'hormone thyréotrope, d'où un état d'hypothyroïdisme qui permet à l'œstrogène de produire des lésions pathologiques sur la glande mammaire. Dans le cas d'hypothyroïdisme expérimental, ce processus s'abrège. L'hypothyroïdisme spontané, en clinique, serait un terrain favorable à l'éclosion de la mastopathie.

Cet ensemble de résultats montre que la déficience de la thyroïde joue un rôle important dans la production, par les hormones stéroïdes, des lésions pathologiques de la mamelle.

RÉSUMÉ

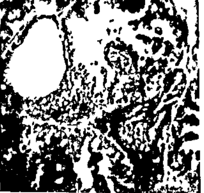
1. Les hormones stéroïdes capables de stimuler la glande mammaire, montrent des différences dans leur action qui

Fig. 10. Différents aspects microscopiques qu'on observe dans les mamelles

épidermoïde; cellules à protoplasme clair et desquamation épithéliale abondante à l'intérieur d'un tube galactophore. ×200.



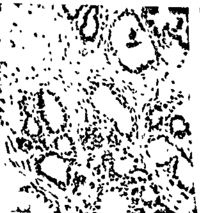
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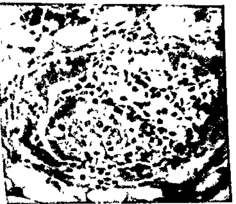
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D



E



F

(Fig. 10)

un état d'hypothyroïdisme ou inhibée par administration de thyroxine.

Dans un état d'hypothyroïdisme expérimental, les androgènes déclenchent rapidement une hyperplasie kystique de la mamelle.

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dépend de leur constitution chimique et de l'espèce animale chez laquelle elles sont administrées. Les œstrogènes et la progestérone provoquent la croissance des tubes et la formation d'acini mammaires, mais l'activité de la progestérone est très faible. Le propionate de testostérone stimule les tubes et les acini pré-existants, mais ne produit ni de bourgeons de croissance, ni d'acini. La synergie qu'on obtient par l'administration simultanée d'œstrogène et de progestérone peut être aussi obtenue par d'autres hormones stéroïdes ayant une action progestative, comme l'acétate de désoxycorticostérone et la testostérone. Le développement le plus rapide et complet de la glande mammaire est obtenu par l'action synergique de faibles doses d'œstrogène et de progestérone.

Les hormones stéroïdes sécrétées par la surrénale ne jouent pas un rôle appréciable dans la croissance normale de la glande mammaire.

Il n'est pas possible, expérimentalement, de mettre en évidence un véritable antagonisme entre œstrogènes et androgènes, sur la mamelle. L'administration associée de ces hormones stéroïdes donne une action synergique.

2. Les hormones stéroïdes n'agissent sur la glande mammaire qu'avec la coopération de l'hypophyse. Il s'agit d'une synergie sur le récepteur. Mais le mécanisme intime de cette action n'est pas encore éclairci. Bien que l'implantation d'hypophyses produise, chez les animaux castrés et hypophysectomisés, une certaine stimulation mammaire, cette action est très renforcée si on associe un stéroïde mammogène. La coopération des hormones stéroïdes et de l'hypophyse est nécessaire pour obtenir un développement complet de la glande mammaire.

3. La thyroïde joue un rôle de protection contre l'action pathologique de certaines hormones stéroïdes. Un état d'hypothyroïdisme expérimental favorise l'apparition d'hyperplasie kystique de la mamelle.

L'hyperplasie kystique de la mamelle, provoquée par l'administration de substances œstrogènes, peut être favorisée par

in the thyroidectomized animal could be produced with a fraction of the minimal effective dose of estrogen in an intact animal, and that

whatsoever.

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DISCUSSION

PULLINGER: Do you find this cystic disease in rats spontaneously? In mice, although you can produce the cystic disease with non-physiological large doses of œstrogen, I never saw it occurring spontaneously in the R-III strain. I have seen it in the Simpson strain. Does this condition develop in mice which are simply thyroidectomized and not treated, as opposed to rats?

CHAMORRO: We have not done these experiments on mice.

GARDNER: I am amazed to find that the testosterone when given with œstrogen has augmented the growth of the mammary gland as much as Dr. Chamorro has described. In our long-term experiments we have accumulated considerable evidence that the mammary growth was impaired by the addition of testosterone in the ratio of 1:1000. We have not, however, used animals of the R-III strain. This has been done primarily using animals of the C_3H strain.

FOLLEY: Do you believe that the effect of thyroidectomy on mammary development in non-spayed females is due to an effect on the metabolism of endogenous œstrogen? Does it mean that in thyroidectomized animals the œstrogen is not inactivated so rapidly as it is in an intact animal?

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in the thyroidectomized animal could be produced with a fraction of the minimal effective dose of œstrogen in an intact animal, and that thyroidectomy does increase the sensitivity, to a given dose of œstrogen in the rat. In clinical observations we have attempted to see whether in [illegible]

whatsoever.

THE EFFECT OF STEROIDS ON THE INCIDENCE OF MAMMARY TUMOURS IN MICE

O. MÜHLBOCK

I AM glad to have the opportunity to discuss with you some experiences we at Amsterdam have had in investigating the effect of certain steroid hormones on the development of mammary cancer in mice. Our experiences do not include all steroid hormones but are mainly concerned with the influence of sex hormones. Before giving the results, I should like to say a few words on the technique of administration. All experiments were made with hormones in the form of pellets of 1–2 mg., administered subcutaneously. Pellets of pure progesterone and pure testosterone propionate were given. Preliminary tests showed that the application of pure œstrone produced too high a mortality; 25 per cent œstrone mixed with 75 per cent cholesterol is tolerated and effective. In order to clarify the results of our experiments, a survey of the strains and hybrids used in the experiments, and the abbreviations of their names, is given in Table I.

Table I

STRAINS AND HYBRIDS EXAMINED

d=Dilute-brown-(Little)	high-cancer-strain
B=C_{57} Black-(Little)	low-cancer-strain
O=O20-Leeuwenhoekhuis	low-cancer-strain
A=A Strong	cancer-strain
dB=F_1 (♀ dilute brown×♂ C_{57} Black)	
dO=F_1 (♀ dilute brown×♂ O20)	

In the first experiments normal females of the d-strain were treated with hormone pellets (Table II). Table II and the next ones are arranged in the following manner: "No." represents the number of animals at the beginning of the

experiment; "No. living at tumour age" includes only those that survived to tumour age, as determined by the age of the first animal in the particular group to develop a tumour. For your convenience the mammary-tumour-incidence is always given as a percentage. "Tumour age" in the next column means the age when tumours were first palpable. "Non-tumour age" means the age at death of non-tumorous mice.

Table II

FEMALES OF THE D-STRAIN TREATED WITH HORMONE PELLETS

	No	No. living at tumour age	Percentage mammary tumour incidence	Tumour age Months	Non-tumour age Months
Virgins untreated		87	59	18	18
Breeders		122	59	15	19
Œstrone at one month of age	35	26	62	14	15
Œstrone first day of life	49	29	45	14	16
Progesterone first day of life	17	15	53	13	14
Testosterone first day of life	24	8	25	18	18

The first group of untreated females acted as controls. The tumour percentage was the same in breeders and virgins; the average tumour age in breeders, however, was three months earlier. Œstrone treatment at the age of one month showed the same percentage of tumour incidence, the tumour age corresponding with that of breeders. Treatment at the first day of life gives the same result. There is no significant difference in the incidence between the two treated groups. The mortality in the treated groups was very high. Treatment with pure progesterone pellets on the first day showed the same effect as treatment with œstrone. As could be expected, testosterone given as pure testosterone propionate

pellets had an inhibiting effect, but the mortality was so high that no safe conclusions could be drawn.

The next experiments were made on males (Table III).

Table III

	No.	*No. living at tumour age*	*Percentage mammary tumour incidence*	*Tumour age Months*	*Non-tumour age Months*
♂d	78	44	4	14	17
♂d castrated .	71	64	14	12	14
♀d virgins untreated . .		87	59	18	18
♂dB . . .	20	19	21	15	21
♂dB castrated . . .	8	8	75	15	20
♀dB virgins untreated		80	67	19	21
♂dO	27	20	40	10	16
♂dO castrated . .	21	21	71	12	24
♀dO virgins untreated		73	93	14	16
♂A castrated .	27	26	46	10	10

Table III shows the results in normal and castrated males treated with œstrone pellets at the age of one month as compared with the incidence of mammary tumours in virgin females. Three groups were investigated: d, dB and dO. *The incidence of cancer in normal males is considerably* lower than in untreated virgin females. This is especially striking in the normal males of the Dilute-brown strain, in which only a few mammary tumours occur. The *mortality of the* treated d-males is rather high. Only 44 of the 78 d-males reached the tumour-age. The average tumour-age in the treated males is earlier than in the virgins.

The first question which arises when determining the cause of these low figures in normal males is whether the mammary gland has been developed sufficiently as a result of the treatment. Therefore in treated normal d-males, whole mount mammary preparations were made of 34 animals after varying periods of treatment. In only 9 per cent could the mammary gland be considered to be satisfactorily developed.

An antagonistic action of the testes is first considered when seeking the cause of the absence of growth of the mammary gland in these males. Gardner found an antagonism between œstrone and testosterone in respect of the development of the mammary gland. We showed this antagonism in the following manner: we used the method of Turner and Lewis to determine the threshold value at which œstrone evokes the first signs of growth in the mammary gland; these first signs are formations of club-shaped swellings at the ends of the ducts; the swellings are accumulations of cells, among which many mitoses are to be found (*Acta Brevia Neerlandica*, 1948, **16**, 1).

Comparison of the threshold values of normal and castrated males shows a considerable difference, especially in males of the d-strain: 1 μg. œstrone in castrated against 50 μg. in non-castrated animals. No such large difference was observed in the two other strains (B and O20) investigated. With the same method it could be shown that testosterone is capable of counteracting the influence of œstrone on the mammary glands.

The next experiment was therefore carried out on castrated males (Table III). In the d-strain the frequency after castration remains low; lower than in castrated males of the A-strain. Experiments on non-castrated males of the A-strain have not yet been completed. However, Bonser has recently proved that the percentage of mammary tumours in the castrates of this strain is higher than in normal males. The results in the hybrids indicate that the incidence is higher in the castrated animals. In the dB-group the difference is significant, but in the dO-group the significance is doubtful.

Whole mounts of the d-strain were examined to determine whether the mammary gland had developed in œstrone-treated castrated males. Seven of the 11 glands examined had developed well. Despite a better development of the mammary gland, the frequency of tumours was not higher.

The next experiments were designed to find a treatment which gives a higher incidence of mammary tumours in

castrated d-males. Firstly we tried progesterone. I think it is agreed at present that progesterone alone may also stimulate the development of mouse mammary gland. I believe that Gardner was the first to prove this some years ago. It is also agreed that progesterone requires much higher dosage than does œstrone. Using the method already mentioned, the threshold value for the first signs of mammary growth in castrated males of the d-strain was determined. Progesterone provokes the same first signs of growth as œstrone does. *The threshold value for progesterone was 500 μg.*, which is 2,500 times that of œstrone (0·2 μg.).

A combination of œstrone and progesterone was tried in castrated Dilute-brown males so as to determine the possibility of producing better mammary development, i.e., a higher tumour frequency (Table IV).

Table IV

MAMMARY-TUMOUR-INCIDENCE IN D MALES CASTRATED AND IMPLANTED WITH ŒSTRONE+PROGESTERONE PELLETS

	No	*No. living at tumour age*	*Percentage mammary tumour incidence*	*Average tumour age Months*	*Non-tumour age Months*
♂d	32	24	4	14	18
Mammary development in 10 whole mounts					
No growth		*Little growth*		*Developed*	
0		2		8	
With Progesterone only					
	No	*No living at tumour age*	*Percentage mammary tumour incidence*	*Average tumour age Months*	*Non-tumour age Months*
♂d	71	58	0	—	16
Mammary development in 19 whole mounts					
No growth		*Little growth*		*Developed*	
1		18		0	

Table IV shows that the expected rise was not produced. It is doubtful if the mammary gland developed better than after œstrone alone. It is therefore not surprising that implantation of pure progesterone-pellets alone remained without effect. Mammary development was only slight.

In an experiment carried out by Korteweg many years ago, in which ovaries were grafted into castrated males of the d-strain, the cancer percentage was higher than after hormone treatment. *This experiment was repeated (Table V).*

Table V

	No	*No living at tumour age*	*Percentage mammary tumour incidence*	*Tumour age Months*	*Non-tumour age Months*	*Percentage still living*
♂d	37	36	64	13	13	22

Dilute-brown males were castrated and grafted with ovaries of their sisters at the age of one month. The cancer percentage was shown to correspond with that of untreated females.

Thus, hormone treatment under the conditions of our experiments cannot equal the effect of grafted ovaries in males of the d-strain.

Hormone treatment in the experiments mentioned so far was started at the age of one month, as it was assumed that the earlier hormone treatment is started, the higher is the frequency of the mammary tumour. The next experiments were done to test the influence of the age at which the hormone treatment was started.

Table VI shows the results in normal males treated at the age of 6 and 12 months. In the d-strain, of course, no results can be expected. *Comparison of tumour frequency in hybrids* treated at one and six months shows the percentage to be little lower when treatment is started later. The average tumour age is later, as was clearly demonstrated in hybrids dO.

Table VI

NORMAL MALES TREATED WITH ŒSTRONE

	Beginning treatment at Months	*No*	*No living at tumour age*	*Percentage mammary tumour incidence*	*Tumour age Months*	*Non-tumour age Months*
♂d	6	25	10	10	16	21
♂d	12	63		0		19
♂d	1	78	44	4	14	17
♂dB	6	41	38	16	17	20
♂dB	1	20	19	21	15	21
♂dO	6	25	18	33	17	22
♂dO	1	27	20	40	10	16

Table VII gives the results of treatment of castrated males at a later age. Of course nothing can be expected from the treatment of d-males. But neither does ovarian grafting at the age of 12 months give rise to tumours. In this experiment the d-males were castrated at the age of one month and at the age of 12 months ovaries were grafted from one month old d-females. These animals showed good mammary

Table VII

CASTRATED MALES TREATED WITH ŒSTRONE

	Beginning treatment at Months	*No*	*No living at tumour age*	*Percentage mammary tumour incidence*	*Tumour age Months*	*Non-tumour age Months*
♂d	6	82		7	18	19
♂d	12	21	—	0		18
♂d grafted with ovaries	12	20	—	0		18
♂dO	6	28	24	29	18	20
♂dO	1	21	21	71	12	24
♂dB	12	38		0		23
♂dB	1	8	8	75	15	20

development, often with hyperplastic nodules, but no mammary cancer was observed. In the group hybrids dO, castrated and implanted with hormone pellets at the age of six months, the tumour percentage found was 29 per cent, as against 71 per cent found when treatment was begun at one month. The average tumour age was six months later. Treatment begun at 12 months gives no cancer in dB-hybrids. Thus treatment begun at a later age than one month results in a decrease in the percentage of tumour incidence.

In the next experiment treatment was started as early as possible, i.e., on the first day of life.

There was no difference in the cancer incidence with treatment begun on the first day of life and treatment begun at one month in female d (see Table II). Table VIII gives the

Table VIII

FEMALE AND MALE Od FOSTERED BY FEMALE d FROM THE EIGHTH DAY OF LIFE

		No	*No living at tumour age*	*Percentage mammary tumour incidence*	*Tumour age Months*	*Non-tumour age Months*
♀litter mates	untreated virgins	32	32	100	13	
	œstrone from the first day	26	11	91	11	9
	œstrone at the age of one month	27	26	89	11	20
♂litter mates	♂ impl œstrone first day of life	24	13	62	8	18
	♂ œstrone one month	17	17	71	11	23

results in male and female hybrids Od. These hybrids are free from the mammary-tumour-agent, therefore they were fostered by a d-female from the eighth day of life. One of three female littermates remained untreated; the second was

treated with œstrone pellets on the first day, and the third with œstrone at weaning age. The same treatment was carried out with male littermates; the first group was treated with œstrone from the first day; the second group received œstrone at the age of one month. Both groups of males were castrated at the age of one month. There was no difference in tumour incidence between the various groups. Comparison of the tumour-age in the females shows no difference whether treatment is begun at the first day or at the first month. Whole-mount preparations of the mammary gland in the females treated on the first day with œstrone showed no development after three weeks; in fact, there was little if any difference with the mammary glands of untreated females of the same age. Perhaps the administration of œstrone to these very young animals gives no mammary development because the hypophysis is not yet functioning.

In the males there is a difference in the average tumour-age between the two groups. Whole-mounts show that there is in the treated group some slight mammary development, which of course is lacking in the untreated males at one month of age.

It remains unexplained why there is a difference in the average tumour age between females and males treated on the first day of life. This matter is still under investigation.

This is a short review of the results of some of our experiments. These results confirm that steroids have an effect on the development of mammary-tumours in mice, but they clearly show that a variety of factors have to be taken into account in the evaluation of these effects.

DISCUSSION

GARDNER: Did you implant just one pellet of œstrone and cholesterol and one pellet of progesterone? How long did these pellets last in the animal?

MÜHLBOCK. Œstrone and cholesterol lasts 1½–2 years, but progesterone varies. After some months you cannot find the progesterone pellets.

BEGG: Have you any evidence that there was still œstrone in the pellets, not just cholesterol?

MUHLBOCK: Yes; after one and a half years I have extirpated the pellets and put them into castrated female mice, and there was mammary growth.

FOLLEY: You used pure progesterone?

MUHLBOCK: Yes.

from the pellets.

MUHLBOCK: But they had been getting some progesterone, because we found development of the mammary gland in practically all of the animals.

FOLLEY: You might get the beginning of development, but you wouldn't get full mammary development unless you had a pretty large dose of progesterone.

of the goat with pellets to get enough progesterone uptake to have any effect on the udder. That's why we have to use injections to administer amounts of about 40 mg. a day.

BEGG How does a tiny pellet last so long?

HERTZ The cholesterol holds it up. What is the concentration of the œstrogen?

MUHLBOCK 25 per cent. I tried some other concentrations, but if

to six weeks.

FOLLEY We have found that 50 mg. pellets of œstradiol in humans will last for over a year.

working as well.

DMOCHOWSKI: I was interested in the very low incidence of mammary cancer in males of strain d In connection with what Dr. Pullinger just mentioned, as we now know that males of high-cancer strains contain the milk factor in their tissues, and that they are susceptible,

then it is the third factor, the hormonal, which does not seem to be effective. Have you any suggestions why the incidence was so low? As far as I am aware, no experiments have been carried out comparing the amount of milk factor in males of various high-cancer strains. ... ? Perhaps the rate of elimina-

experiments carried out in the United States and in this country, the incidence of mammary cancer following œstrone treatment is always very high in males of the high cancer strain and comparable to the incidence in females of the same strain.

MÜHLBOCK: *I will deal with your second point first.* In the hybrids I found practically the same incidence in females and treated males. However, Dr. Bonser, who has treated A Strong male mice, has found only one or two mammary cancers after treatment. I think that probably in this d-strain the margin between the effective dose and the toxic dose is too narrow. Maybe I would get a higher incidence if I gave more œstrone, but the mortality would be still higher than it is with 25 per cent.

HUGGINS. To what do you attribute the increased efficiency of the ... in producing cancer in the d males?

DMOCHOWSKI I am not aware that this has been done, but I do not see any reason why they should be inactivated.

HADDOW: *You have assumed, Dr. Muhlbock, that the influence is not direct?*

MÜHLBOCK: Yes. Mammary-tumour-agent is present in castrated and in pregnant female mice, although there is much more hormone in the latter.

ASTBURY: But no one has simply tested an extract of these virus particles to see if they're directly influenced chemically by these hormones?

... œstrone is

ild

ay

GARDNER: Yes, that is the hypothesis [illegible]

[illegible]

uniquely different from the other mice that were mentioned in that they do show adrenal tumours after the gonads have been removed for a long time, whereas I know the C_{57} does not. In the males of the ce strain, they have occasionally found a mammary tumour as well as the adrenal tumour.

MÜHLBOCK: Our subline of the DBA strain does not show any adrenal tumours.

HORMONAL RESPONSES OF MAMMARY TUMOURS IN MICE

L. F. FOULDS

I BECAME involved in the study of mammary tumours by following a procedure designed specifically to avoid them. I bred hybrid mice, using C_{57} black females and R III males, and found an appreciable number of tumours in the F_1 hybrids although they ought not to have had any milk factor from their C_{57} black mothers. I thought it might be interesting to see if there was anything peculiar about the tumours as shown by transplantation, and I made a few experiments to see if milk factor was present.

I will pass over these second experiments very quickly. They show that milk factor is present. Where it comes from is another matter, which I don't propose to go into. I should like to emphasize now that all the mice to which I shall have occasion to refer this morning, so far as all the evidence goes, do possess the Bittner milk-factor.

One of the experiments consisted in breeding from those F_1 mice which developed mammary tumours. I have inbred from some of those mice now for 12 or 13 generations, and the offspring provide many of the tumours which I shall describe. The remaining mice are the reciprocal hybrids of the F_1 generation, that is, they have R III mothers and C_{57} black fathers. Most of the observations have been made on one or other of these hybrids.

The tumours, when transplanted, grew very well in normal female mice. They did not grow at all, or they grew only after a long delay, in intact male mice and they did not grow in castrated male mice. In males which received pellets of stilbœstrol in cholesterol, the tumours grew as well as in normal females. In one or two experiments the pellets were

removed from males after the transplanted tumours got well going. Sometimes there was a slight arrest of growth, but it was temporary, and eventually the tumours went on growing. So the action of œstrogen seemed to be mainly on the initial take of the tumour rather than on its continued growth.

There were two other bits of evidence for hormonal action on these tumours. Some of them were very conspicuously milky. When they were cut they oozed a milky or even creamy fluid from their surface and they often had milky cysts. This was found only in pregnant females or in œstrogenized males; in males from which the œstrogen pellets had been removed the tumours were no longer milky. It occurred in a minority of the tumours which showed the difference in transplantability in males and females. The second point is that tumours of two strains grew during pregnancy and tended to regress, though not completely, after parturition, which is the reverse of the usual experience of the effect of pregnancy on transplanted tumours. One other thing I should say about these phenomena is that they were temporary; when one went on transplanting, then sooner or later the tumours grew equally in males and females. Sometimes this occurred after the first or second passages, sometimes after half a dozen or more.

When the experiments on transplantable tumours had got to this stage, spontaneous tumours began coming up in the breeding experiments, and I did not follow up the transplantation experiments but concentrated on spontaneous tumours. So the tumours I am going to describe now are spontaneous tumours growing in their original hosts.

When tumours began appearing in the breeding experiment, it was noticed that a tumour found one week couldn't be found the next week. When that had happened a few times I looked into the matter and found that about two-thirds of the tumours grew during pregnancy and regressed very rapidly after parturition. The tumours were measured regularly, and their size was plotted against time. The course of the familiar type of spontaneous mammary tumour, which

I call "unresponsive," as it grows without any regard to pregnancy or parturition, *was represented by an approximately* straight line. The other tumours, which were in a majority and which I call "responsive" tumours, were represented by waves whose peaks *corresponded approximately with the* times of parturition in successive pregnancies. There were two main types of "responsive" tumour. In one type, the tumours reached about the same peak size in successive pregnancies and regressed, often completely, between pregnancies. In the other type, the peak size increased steadily in successive pregnancies and regression between pregnancies was sometimes complete, but often only partial. Regression after parturition was often remarkably abrupt. Tumours of substantial size (e g., $1 \cdot 5 \times 1 \cdot 0$ cm.) often disappeared within 24 hours. The responsiveness sometimes persisted through many pregnancies over a long time. Thus, in a mouse under observation for 21 weeks, one tumour reached the same size in six successive pregnancies and regressed after parturition, whilst another grew gradually, showing a little fluctuation about the time of each parturition. Sometimes there was a much bigger swing and tumours reached a substantial size, the maximum being about $2 \cdot 5$ cm. in diameter, whilst still responsive to pregnancy. If breeding were stopped by removing the male, the tumours disappeared and, as a rule, they did not come back again until the male was brought back and breeding resumed. Then they promptly reappeared. *Some tumours maintained the same type of behaviour* throughout their clinical course, but others changed their course often abruptly; in particular, tumours which previously *had grown only during pregnancy* lost their dependence on the stimulus of pregnancy and then grew steadily and progressively without regard to reproductive activity. When, as was common, multiple responsive tumours were present, the change to unresponsive behaviour occurred in only one of them. For example, two tumours appeared simultaneously in one mouse and a third developed later. The three tumours followed parallel courses, growing during pregnancy and

regressing thereafter until the ultimate pregnancy; then the three tumours grew parallel up to the time of parturition, whereupon two tumours promptly regressed, whereas the other continued to grow steadily. The tumour that continued to grow was one of the pair that had appeared first. I think that this and other similar observations can only mean that a change occurred in one of the tumours; had the whole animal changed, all the tumours would have been affected equally. I have described the change as "progression" by which I understand an irreversible qualitative change in the tumour itself, whereby the tumour, previously responsive to pregnancy, becomes unresponsive and grows progressively until it kills the animal, whether the animal breeds or not. Sometimes a tumour does recur in the absence of pregnancy. For example, the male was removed from a mouse with three tumours and no more pregnancies occurred during the period of observation. The three tumours disappeared; one of them recurred after three weeks and grew steadily and progressively without any stimulation. That tumour had changed from the responsive to the unresponsive type, irrespective of pregnancy. My suspicion is that, in general, pregnancy is not essential for the occurrence of progression although it often brings it to light.

I can't go into details very closely, but the peak of growth was reached, as a rule, during the last two or three days of pregnancy. Regression began, I suspect, a little before the actual time of parturition—it is difficult to be certain—but it was pronounced within the first 24 hours. Recurrence was usually obvious during the second half of the next pregnancy, but I think it probably started considerably before then, and there were some indications that the recurrence possibly started during the first week of pregnancy.

One of the main problems to be followed up is: what is the hormonal mechanism involved? My colleague, Mr.

I call "unresponsive," as it grows without any regard to pregnancy or parturition, was represented by an approximately straight line. The other tumours, which were in a majority and which I call "responsive" tumours, were represented by waves whose peaks corresponded approximately with the times of parturition in successive pregnancies. There were two main types of "responsive" tumour. In one type, the tumours reached about the same peak size in successive pregnancies and regressed, often completely, between pregnancies. In the other type, the peak size increased steadily in successive pregnancies and regression between pregnancies was sometimes complete, but often only partial. Regression after parturition was often remarkably abrupt. Tumours of substantial size (e.g., $1 \cdot 5 \times 1 \cdot 0$ cm.) often disappeared within 24 hours. The responsiveness sometimes persisted through many pregnancies over a long time. Thus, in a mouse under observation for 21 weeks, one tumour reached the same size in six successive pregnancies and regressed after parturition, whilst another grew gradually, showing a little fluctuation about the time of each parturition. Sometimes there was a much bigger swing and tumours reached a substantial size, the maximum being about $2 \cdot 5$ cm. in diameter, whilst still responsive to pregnancy. If breeding were stopped by removing the male, the tumours disappeared and, as a rule, they did not come back again until the male was brought back and breeding resumed. Then they promptly reappeared. Some tumours maintained the same type of behaviour throughout their clinical course, but others changed their course often abruptly; in particular, tumours which previously had grown only during pregnancy lost their dependence on the stimulus of pregnancy and then grew steadily and progressively without regard to reproductive activity. When, as was common, multiple responsive tumours were present, the change to unresponsive behaviour occurred in only one

bulbous extremities. There is usually a lumen in the columns, but, as a result of the very great epithelial proliferation, the lumen sometimes becomes quite inconspicuous. At the height of their growth these epithelial masses contain many mitotic figures, and they are separated from each other by a very cellular, very loose and vascular connective tissue with no collagen. Soon after parturition a breaking down of these epithelial masses is seen. The tubes are now lined only by a single layer of epithelium. The bulk of the epithelium is desquamated into the lumen. As I can't find much sign of phagocytosis or cellular reaction either in or around these tubules, I presume that the desquamated masses are extruded by way of the mammary ducts. When the epithelial degeneration begins, the connective tissue also changes and collagen forms between the epithelial columns. Two or three days after parturition, we find empty tubules with a single-layered epithelial lining, and a certain amount of shrinkage, compression, and collapse of the tubules and fibrous collagenous connective tissue between them. This process goes on to produce a nodule with a peripheral part of quite dense fibrous tissue with collapsed, compressed, epithelial tubules and often a central area of loose fatty tissue containing some ducts.

Many of the tumours which regressed after parturition disappeared completely; one couldn't find them either by the naked eye or with a dissecting microscope. What happens to those I cannot say, but possible transitions were found. For example, at the site of a tumour which had regressed in a mouse which had not been pregnant for about three months previously, there was a fibrotic plaque which was obviously incomplete; in the animal it looked a distinct well-outlined plaque; histologically, patchiness of the fibrous area was conspicuous. In another mouse whose tumour had regressed about seven months previously, one could see by the naked eye a fairly distinctly outlined plaque, but histologically there was only a thin rim of fibrous tissue around the edge, enclosing tissue resembling normal breast. It is possible to imagine this process going on a little further so that one would

against in dosage, and the balance in dosage of different hormones. So far, we have no clear indication that it is either a purely œstrogenic effect or a progesterone effect, or even a combination, although I must admit we haven't enough information with balanced doses to draw firm conclusions.

The unusual behaviour on transplantation and the growth in relation to pregnancy were quite unexpected and raised the question whether the tumours differ in some way from those with which we are familiar in most mice. The final big tumour which kills the mouse, so far as I can see, is no different from the ordinary mammary tumours of inbred or cross-bred mice which have been described in the last 40 or 50 years. The early stages do seem to be different. Unfortunately, it was not possible to keep the histological work in line with the experimental observations or to do whole-mounts on the breasts, and, to tell the truth, I did not foresee at the time how useful they might have been. All that I can say now is that the hyperplastic nodules which have been described by Dr. Gardner and others, and which are common in most high tumour strains, are, at least, not conspicuous in these mice, and I don't think the tumours develop from them. The first sign I see is a flat disk or a plaque in the subcutaneous tissues. A characteristic feature is that there are usually one or more fairly normal-looking ducts in the centre, sometimes even a fairly distinct division between a "medulla" and a "cortex," with tubules often radiating from the centre towards the periphery. Some bigger tumours are made up of several lobules of the kind which make up the small one. The histological appearances are extremely varied and complicated and I do not pretend to have analysed any but the simpler ones.

There is one kind of tumour, however, in which the sequence of changes is, I think, fairly convincing. The small tumours near the end of pregnancy and at about the peak of growth, have a centre occupied by small ducts and columns of epithelial tubules radiating outwards. There is a certain amount of branching of these radiating columns, which often have

It seems to me that the essential histological feature of these tumours is that the type of growth is essentially organoid; it is not a mere cell proliferation. The growths imitate, to a greater or lesser extent, the development of the normal breast and, to some extent, though not exactly, they react to similar hormonal conditions. I would point out one difference, that immediately after parturition the responsive tumour is quite out of step with the surrounding normal breast: the normal breast is full of secretion and active whereas the tumour is already, within a few hours of parturition, well on the way to disappearance.

I rather suspect that the phenomena I have described may correspond to something which occurs only occasionally in other strains of mice; what is rare in most strains is the usual and commonplace feature in these particular hybrid strains. I am less interested in their application to the particular problems of mammary tumours in mice than to the more general problems of responsiveness to hormones and progression from a responsive to an unresponsive state, both of which I think are highly relevant to our discussion to-day.

DISCUSSION

HADDOW: There is no doubt this is a very important observation. [illegible]

pregnancy, whereas in any case it would have appeared about that time.

FOULDS. The time of recurrence in our mice was quite erratic. It varied by some months.

HADDOW: Willis, I think, described a case of the development of a mammary cancer in a young woman during pregnancy. For other reasons, the pregnancy was terminated and the mammary carcinoma completely regressed and remained absent until a subsequent pregnancy in which it reappeared.

not be able to see anything at all by the naked eye. In early pregnancy, the sequence of changes which I described for regression goes in the reverse direction: the tubules begin to swell up, the fibrous tissue loosens, the collagen disappears and the tumour reverts to the active plaque which I described before.

Most of these tumours regress whether or not the mouse nurses its young. A few, however, do not regress while the animal is nursing, though they may regress later, and some at least of these during the nursing period have the structure of secreting adenomas. There is also a trace of the architecture which I described in the other tumours, with branching tubular duct-like formations.

There is a little evidence about the mechanism and the histological basis of what I call "progression," that is the change of properties of the tumour during observation. In some regressed tumours the regression is patchy, with survival of some small patches of epithelial tubules which have not regressed in step with the bulk of the nodule; or whilst the bulk of a tumour is degenerate and becoming fibrous, one lobule is obviously different and retains the structure which we normally find during pregnancy. This I interpret as altered tissue which probably goes on to form a progressively growing, unresponsive tumour. In some regressed plaques there is sometimes a central, sometimes a peripheral nodule of what is recognizable as fairly typical mammary tumour. These nodules again, I think would have given rise to a progressively growing, unresponsive tumour. The fact that these unresponsive tumours become apparent very often at the end of a pregnancy may, I think, be due to a vascular effect. The nodules from which they start in non-pregnant mice are enclosed sometimes in a quite dense fibrous tissue and may have a very poor blood supply. When pregnancy occurs and the whole surrounding tumour becomes better vascularized, then the nodules get going, and once they have acquired a good blood supply they continue to grow indefinitely.

closely related to Compounds E and F. On the other hand, there appear in the urine some new steroids whose nature is not known.

What experience has been accumulated on œstrogen treatment of human males? Somebody mentioned that there are more than half a dozen males with prostate cancer who have developed breast tumours under œstrogen treatment.

HADDOW: I think there are four papers describing development of breast cancer in the male after œstrogen treatment.

HUGGINS: It is not certain whether it's breast cancer or prostatic cancer which has metastasized to the breast.

and cervical cancer. Perhaps a number of these people who have been treated with œstrogens for prostatic cancer clinically presented their prostatic cancer before they presented their second primary breast cancer. I don't think you can jump to the conclusion that there is a cause-effect relationship.

The bone metastases did not have a high acid phosphatase content. It seemed to be coming from the breast.

as to the recurrence of these tumours. For the last two years we have had an experiment set up on similar lines to those of Dr. Foulds.

FOULDS: One transplanted tumour was

Its presen

offspring

incidence

GARDNER: I have been extremely interested in this work which Dr. Foulds has described. On one occasion I saw, I think, three tumours [illegible] after parturition. And, [illegible] ith [illegible] of [illegible] at- [illegible] idy [illegible] ant [illegible] ous [illegible] em [illegible] the [illegible] his tissue. How does the metabolism differ from that in the mammary glands that do not disappear? The histological appearance seems to be the same as in human tumours that disappear under œstrogen treatment.

HERTZ: It is paradoxical that the histology of tumours disappearing under intensive œstrogen administration should be compared to these, which are regressing in the period of withdrawal of the high hormonal level of pregnancy.

HADDOW: We had a few very striking cases at the Cancer Hospital of very active clinical regression on withdrawal of œstrogen.

HERTZ: We've seen that in cases that I'll talk about tomorrow, in which we continued to get very marked degrees of regression after stopping œstrogen in older patients with breast cancer who were no longer responding to therapy.

BEGG: Clinically, the œstrogen regression would depend on whether they are pre- or post-menopausal, wouldn't it?

HERTZ: That is a generalization that has been made, but I don't know if it's too well established What do you think, Dr. Haddow?

HADDOW: I think it's part of the truth, but not all. In the early days we had a joint clinical trial by about 12–14 people, all of whom worked independently for about a year and then came together. About half of these investigators had had this very distinct impression, and when they all came together it seemed to be much more than that, although not the whole truth.

BEGG: Won't a woman with breast cancer have a flare-up during pregnancy?

HADDOW: Apart from any specific effect, the question of blood supply is important.

DOBRINER: Does testosterone have any effect on the tumours?

FOULDS: We haven't tried testosterone. The sort of experiment we've been doing is to remove the males, let the tumour regress, and then try to bring it back artificially.

DOBRINER: It is very interesting that you have a host-tumour [illegible] is dependent on the hormonal environ- [illegible] nal changes in mice [illegible] there are two major

PART III

STEROIDS IN CANCER THERAPY

ANTI-TUMOUR ACTIVITIES OF STEROIDS IN ANIMALS

C. CHESTER STOCK

THIS report includes the studies of several of my associates in the Experimental Chemotherapy Division of the Sloan-Kettering Institute and represents only one of a number of investigations on steroids being conducted at Sloan-Kettering. The Chemotherapy Division programme* includes tests of a number of steroids against the chick embryo by Dr. Karnofsky (Karnofsky, Stock and Rhoads, 1950), against a variety of transplantable tumours in mice and rats by Dr. Sugiura (Sugiura, Stock, Dobriner and Rhoads, 1950), and against mouse leukæmia by Dr. Burchenal (Burchenal, Stock and Rhoads). The mouse leukæmia studies will be presented by Dr. Burchenal at this conference. Clinical and metabolic studies will be reported by Dr. Dobriner. Other aspects of the Institute's biological studies on steroids include Dr. Woolley's investigations on the influence of steroids on organs and tumours in mice, and Dr. Money's studies on steroid-induced changes in the rat and the influence of steroids upon the uptake of radioactive iodine by the thyroid.

The classical experimental and clinical studies of the influence of steroids on tumours by Gardner (1947), Huggins (1942-1946), Nathanson (1947), Haddow (1944), and Adair

*We wish to acknowledge the valuable assistance of Dr. Konrad Dobriner *in this programme.*

[illegible]

carry milk factor obtained somehow or other, and the tumour-free F_1 animals, which are in the majority, do not.

DMOCHOWSKI: I think that the picture is even more complicated. I believe that our observations agree with those of Dr. Andervont from Washington, that not all of these tumours, as far as biological tests are concerned, contain the milk factor. We have 20 at the moment that are being tested biologically, and I can say for certain that some of [illegible]

[illegible]

breeding. It seems to us that forced breeding may produce two types of tumour, one which contains the milk factor, and the other which does not seem to possess it. Experiments are being carried out at the moment to elucidate this point.

(1946, 1949) are well known. These and other published reports have been excellently supplemented by the studies presented at this conference.

Our results with cortisone were foreshadowed by those of Heilman and Kendall (1944) on the inhibition and regression of a mouse lymphosarcoma, and by Murphy and Sturm (1944) on the inhibition of the development of transmitted leukæmia in rats by adrenal cortical extracts. Clinical studies with cortisone and ACTH were under way in patients (Pearson, Eliel, Rawson, Dobriner and Rhoads, 1949) at the time our experiments with steroids on transplanted tumours in mice and rats were initiated. We were interested in determining:—

(1) Whether the lymphosarcomas in our chemotherapy programme, under the test conditions employed, would respond to cortisone.
(2) Whether types of tumours other than those of lymphoid origin would be affected.
(3) Whether there are differences in patterns of activity of different steroids on different tumours.

It was soon apparent that some steroids would be available in amounts too limited even for preliminary trials in the animal. As a result, studies were initiated in the chick embryo and, since cortisone showed a marked activity against the embryo, all available steroids have been screened in the chick embryo (Karnofsky *et al.*, 1950). The experiments with cortisone revealed such striking effects that a discussion of them and the results with other steroids seems warranted prior to a discussion of the activities of steroids against abnormal growth.

Landauer (1947) first reported that an extract of adrenal cortex would inhibit the growth of the chick embryo. In Karnofsky's experiments cortisone acetate has exerted a marked inhibitory effect on the growth of the chick embryo. Initially, aqueous suspensions of 2–4 mg. of steroid were injected into the yolk sac of the four-day-old embryo. The

embryos surviving to 18 days showed moderate to severe stunting with generalized baldness. The effect was explored in more detail and a range of growth inhibition was found. The range of effects has been divided into four categories as illustrated in Fig. 1. The embryos dying before the 18th day are small, pale and œdematous, have unusually large eyes in relation to body size, and are often eviscerated. Those surviving to 18 days present a characteristic syndrome. The embryo is small; the yolk sac and chorioallantoic membrane have not formed completely, and there is consequently free yolk and albumin present which is not enveloped by membranes. The embryo adopts a characteristic, curved position and the amnion is drawn tightly around it. The embryo is pale and the feathers are practically absent; evisceration is common. In Karnofsky's experience other types of chemical agents have not produced a similar stunting. At lower doses are seen intermediate changes, consisting of diminished feather formation with baldness, clubbed feathers, and varying degrees of stunting in body growth. Embryos showing the intermediate effects may survive until 21 days but they do not hatch.

The effect of cortisone on the embryo was quantitated at four days and at eight days for yolk sac injections and at eight days for the chorioallantoic route of administration. In the latter the maximum effect was obtained with 2 mg. of cortisone acetate and larger amounts were required by the other route (Table I). The relationship of time of dosage to the effect was studied and the data in Table II indicate that inhibition has been obtained by both routes of administration until the 12th day. The physiological significance of this observation may be clarified by histological studies still in progress.

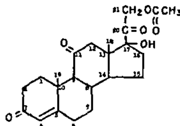
The assay of steroids for cortisone-like activity has been developed as follows:—

The steroid solution or suspension is placed on the chorioallantoic membrane of the eight-day-old developing chick embryo. Ten days later the embryo is sacrificed and graded

CONTROLS	STAGE I	STAGE II	STAGE III	STAGE IV
WGT. 16–21 G	SACRIFICED, 18 DAYS INCUBATION			
	SLIGHT FEATHER CHANGES SLIGHT STUNTING USUAL WGT 13-16 G	MODERATE BALDNESS CHIEFLY ON HEAD CLUBBED FEATHERS MODERATE STUNTING USUAL WGT 10-14 G	SEVERE GENERALIZED BALDNESS SEVERE STUNTING USUAL WGT 8-11 G	"CORTISONE SYNDROME"

Fig. 1 Range of effects produced by cortisone in the chick embryo

for effect. With this technique a number of steroids have been tested with the results presented in Figs. 3, 4, 5 and 6. Fig. 3 shows the approximate minimal amounts of compounds E, F, A and corticosterone needed to achieve definite inhibition of the embryo. The greater effectiveness of Compound F is striking. In Fig. 4 the activities of Compound S and DCA



11-DEHYDRO-17-HYDROXYCORTICOSTERONE
ACETATE

FIG. 2. Formula for cortisone showing numbering system for reference on steroids listed in the tables.

11-DEHYDRO-17α-HYDROXY CORTICOSTERONE
COMPOUND E
ACETATE + 0.5 mg.
FREE

17α HYDROXY CORTICOSTERONE
COMPOUND F
+ 0.02 mg.

11-DEHYDRO CORTICOSTERONE
(KENDALL'S COMPOUND A)
ACETATE + 0.2 mg
FREE

CORTICOSTERONE
+ 0.2 mg

FIG. 3. Formulæ of compounds with approximate minimal amounts required to inhibit the development of the chick embryo.

Table I

AVERAGE RESULTS OF EFFECTS ON CHICK EMBRYO FROM VARIOUS AMOUNTS OF CORTISONE ADMINISTERED BY SEVERAL ROUTES
ACTIVITY OF CORTISONE ACETATE IN THE CHICK EMBRYO

Dose mg/egg	4-day yolk sac		8 day yolk sac		8 day chorioallantois	
	Embryo effect (1-4, average)	Weight, g (average)	Embryo effect (1-4, average)	Weight, g (average)	Embryo effect (1-4, average)	Weight, g (average)
	(Sacrificed at 17-18 days)					
0·2					0(3)	16·9
0·5	+0 8(5)*	14 5	0(4)	17·4	+2·7(7)	10·9
1 0	+1 3(21)	13 0	+1 0(4)	13 8	+2·5(2)	10·5
2 0	+2 4(19)	10 8	2 8(11)	8 8	+4·0(8)	5·2
4 0	+4·0(8)	10 1	4·0(3)	4·4		

*Number of embryos.

The effects are graded from 1 to 4 as presented in Fig 1.

Table II

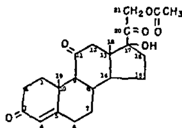
ACTIVITY OF 2MG/EGG OF CORTISONE ACETATE INJECTED INTO THE YOLK SAC AND ON CHORIOALLANTOIC MEMBRANE (0-15 DAYS OF AGE)

Time of injection Days	Route			
	Yolk sac		Chorioallantois	
	Embryo effect (1-4, average)	Weight, g. (average)	Embryo effect (1-4, average)	Weight, g (average)
	Sacrificed 17-18 days			
0	+2·1(6)†	10·0	—	—
2	+2 1(6)	11·3	—	—
4	+1 8(7)	10·7	—	—
6	+2 1(5)	10·0	—	—
8	+2·8(8)	8·4	+4·0(6)	5 2
10	+2 2(9)	10·0	+3 8(4)	7·4
12	+3 1(14)	7 1	+2 6(9)	7·8
15	0(9)	17·5*	0(9)	15 8*
Controls	(16 to 21 g.)			

*Sacrificed, 19 days
†Number of embryos

The effects are graded from 1 to 4 as presented in Fig 1

for effect. With this technique a number of steroids have been tested with the results presented in *Figs. 3, 4, 5 and 6.* Fig. 3 shows the approximate minimal amounts of compounds E, F, A and corticosterone needed to achieve definite inhibition of the embryo. The greater effectiveness of Compound F is striking. In Fig. 4 the activities of Compound S and DCA



11-DEHYDRO-17-HYDROXYCORTICOSTERONE
ACETATE

FIG. 2. Formula for cortisone showing numbering system for reference on steroids listed in the tables

H2C-OH
C=O
OH

11-DEHYDRO-17α-HYDROXY CORTICOSTERONE
COMPOUND E
ACETATE + 0 5mg.
FREE

H2-C-OH
C=O
HO
OH

17α-HYDROXY CORTICOSTERONE
COMPOUND F
+ 0 02mg

H2-C-OH
C=O

11-DEHYDRO CORTICOSTERONE
(KENDALL'S COMPOUND A)
ACETATE + 0 2 mg
FREE

H2-C-OH
C=O
HO

CORTICOSTERONE
+ 0 2 mg

FIG. 3. Formulæ of compounds with approximate minimal amounts required to inhibit the development of the chick embryo.

are compared. It is of interest that DCA has a definite activity although it lacks an 11-oxygen function. The markedly decreased activity of Compound S is worthy of note. With progesterone (Fig. 5) there appears to be little difference due to the presence of the 17-OH group, nor has the activity been lost with the loss of the 21-OH group. In

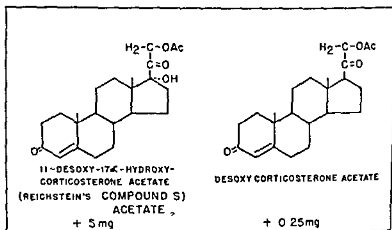


FIG. 4.

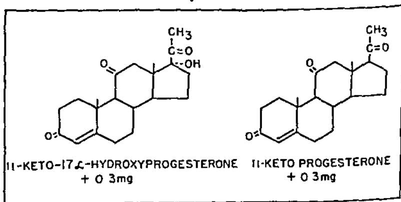


FIG. 5

Fig. 6 it appears that several compounds of the progesterone and Δ^5-pregnenolone series have an activity quantitatively similar to that of Compound S. Over twenty other steroids have been tested at 5 or 10 mg. levels without showing any

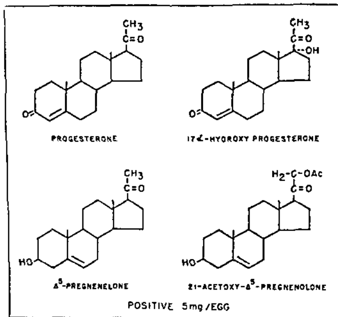


FIG. 6.

FIGS. 4, 5, and 6. Formulas of compounds with approximate minimal amounts required to inhibit the development of the chick embryo.

inhibition of the embryos. In Table III these are listed by name and grouped according to certain common aspects of structure.

In addition, a group of C_{19} steroids were without inhibitory effects. Included were testosterone, adrenosterone, Δ^4-androstenediene-3,17, 17-ethinyl testosterone, $\Delta^{4,6}$-androstadiene-3,17 and 2-acetoxy testosterone acetate.

Table III

STEROIDS INACTIVE AS TESTED AGAINST THE CHICK EMBRYO

Compound	Max. dose level tested, mg/egg
Compounds with an 11 oxygen function and a saturated A and B ring	
11-keto-pregnanolone acetate	− 5·0
3, 11, 20-triketo-4, 21-diacetoxy,17-OH pregnane	− 5·0
Dihydro-cortisone acetate	−10·0
Compounds without an 11 oxygen function and a saturated A and B ring	
Allo-pregnanolone	−10·0
Allo-pregnanediol, 3β, 17α-one-20-acetate 3 (Comp L)	− 5·0
Pregnane-3α, 12α, 21-triol-2p-one, 21 acetate	− 5·0
A compound without an 11 oxygen function and an unsaturation at C_1	
Δ^{1}-17 hydroxyprogesterone	− 5·0
Compounds without an 11 oxygen function and no carbonyl group at C_{20}	
[illegible]	− 8·0
[illegible]	−10·0
[illegible]	− 7·5
[illegible]	− 5·0
[illegible]	− 5·0
[illegible]	− 5·0
[illegible]	− 5·0
A compound without an 11 oxygen function and unsaturated at C_{16}	
$\Delta^{5,16}$-pregnadiene-3β-ol-20-one acetate	− 5·0

To sum up, certain steroids have been found to inhibit chick embryo development at the following levels:—

·02 mg.	Compound F
0·2–0·5 mg.	Corticosterone
	Kendall's Compound A
	Cortisone
	11-ketoprogesterone
	11-keto-17-hydroxy-progesterone
	DCA

5 mg. . . . . . Reichstein's Compound S
progesterone
pregnenolone

The effects observed differ from those caused by X-rays and those caused by the antagonists of folic acid and other vitamins; however, the exact nature of the activity is yet to be determined. It is believed that this will offer a useful tool for detecting small amounts of certain biologically active steroids, though the steroids active against the chick embryo may not duplicate those showing any other type of biological activity. It will be seen that the number of steroids active against certain mouse tumours is more restricted than the number active against the chick embryo.

The studies of the action of steroids against the chick embryo are being extended to include newly hatched chicks (2–3 days). So far, there have been observed inhibitions in weight gain, measured at 10 days, a pronounced effect by compound F, slight effects by DCA and cortisone, some lesser activity from Compound S, progesterone, and testosterone and none from dihydro- and tetrahydro-cortisone compounds A and L, and ACTH (Karnofsky, unpublished, data).

The remainder of the report consists of the studies of the action of steroids against transplantable tumours in animals. The technique has consisted of daily subcutaneous injections for one week of saline suspensions, starting one day after subcutaneous implantation of the tumour implant. The tumours are measured in two diameters by calipers one week later and at subsequent weekly intervals. The results obtained with cortisone against a variety of tumours is included in Table IV. The maximum tolerated dose has been 37·5 mg./k/day (0·75 mg./mouse/day) and it has shown delayed complicated metabolic side effects. The strongest inhibitions have been obtained with the lymphosarcomas and osteogenic sarcomas. Mammary adenocarcinoma EO 771 has been inhibited moderately, while sarcoma 180 has not been affected

Table III
STEROIDS INACTIVE AS TESTED AGAINST THE CHICK EMBRYO

Compounds with an 11 oxygen function and a saturated A and B ring	
	Max dose level tested, mg /egg
11-keto-pregnanolone acetate	— 5·0
3, 11, 20-triketo-4, 21-diacetoxy,17-OH pregnane	— 5 0
Dihydro-cortisone acetate	—10·0
Compounds without an 11 oxygen function and a saturated A and B ring	
	Max dose level tested, mg /egg
Allo-pregnanolone	—10 0
Allo-pregnanediol, 3β, 17α-one-20-acetate 3 (Comp. L)	— 5·0
Pregnane-3α, 12α, 21-triol-2p-one, 21 acetate	— 5·0
A compound without an 11 oxygen function and an unsaturation at C_1	
	Max. dose level tested, mg /egg
Δ^1-17 hydroxyprogesterone	— 5·0
Compounds without an 11 oxygen function and no carbonyl group at C_{20}	
	Max dose level tested, mg /egg
Δ^4-pregnene-triol one	— 8 0
3-keto-Δ^4-pregnane-17β-20, 21 triol	—10·0
[illegible]	— 7·5
[illegible]	— 5·0
[illegible]	— 5·0
[illegible]	— 5·0
[illegible]	— 5·0
A compound without an 11 oxygen function and unsaturated at C_{16}	
	Max dose level tested, mg /egg
$\Delta^{5,16}$-pregnadiene-3β-ol-20-one acetate	— 5 0

To sum up, certain steroids have been found to inhibit chick embryo development at the following levels:—

·02 mg.	Compound F
0·2–0·5 mg. . . .	Corticosterone
	Kendall's Compound A
	Cortisone
	11-ketoprogesterone
	11-keto-17-hydroxy-progesterone
	DCA

aspects of these compounds are listed in Table V. Table VI presents similar details for a group of steroids which have not inhibited the lymphosarcoma even though most of them have been tested at 5–10 times the maximum dose level for corti-

sone. Although the inactive compounds in Table VI show a number of variations in structure, all of them lack an 11-oxygen function. So far, it appears that the active steroids require an 11-oxygen function, and a 3-keto group with the Δ^4 conjugated unsaturation. The 17-hydroxyl group is not essential but appears to increase the effectiveness. A 20-keto

Table IV

EFFECT OF CORTISONE ON VARIOUS TUMOURS IN MICE

Dose mg/kg/day	Sar 180	Sar T241	Sar M 4 337	Car. 1025	EO 771	H-P Mel	WOS	ROS	PLS	MLS
37 5	−	+	−	±	+		++	++	++	++
25·0								++	++	++
12 5								±	+	++

− indicates no effect, ± indicates slight inhibition, + indicates moderate inhibition; ++ indicates marked inhibition

EO771	=	Mammary Adenocarcinoma EO771
H-P mel	=	Harding-Passey Melanoma
WOS	=	Wagner Osteogenic Sarcoma
ROS	=	Ridgway Osteogenic Sarcoma
PLS	=	Patterson Lymphosarcoma
MLS	=	Mecca Lymphosarcoma

at the maximum tolerated dose. The degrees of inhibition of the lymphosarcomas and osteogenic sarcomas are illustrated in Figs. 7 and 8. In the case of the lymphosarcomas an increase in survival time is also noted. Thus far, the histological studies of the inhibited tumours have not revealed more than a mixture of viable tumour cells and those with degenerating nuclei.

The results with cortisone encouraged the study of numerous steroids in the spectrum of tumours with emphasis upon the lymphosarcomas and osteogenic sarcomas. These studies have offered some information on the relationship of steroid structure to inhibition of the lymphosarcoma (Stock, 1950; Sugiura *et al.*, 1950). Compound F is nearly as active as cortisone, whereas two to three times as much compound A is required for comparable activity. 21-desoxycortisone has a very slight activity, probably no more than one-twentieth to one-fiftieth that of cortisone. Dihydro- and tetrahydrocortisone have shown no activity. The structural

Table V

STRUCTURAL ASPECTS OF STEROIDS TESTED AGAINST MOUSE LYMPHOSARCOMA

Compound	Dose mg/kg/day	Activity	11-Keto	11-OH	Δ^4-3-Keto	20-Keto	3-OH	17-OH	21-OH
Cortisone	37·5	+	+	−	+	+	−	+	+
Compound F	37·5	+	−	+	+	+	−	+	+
Compound A (17-desoxy cortisone)	125	+	+	−	+	+	−	−	+
21-desoxycortisone	375	− to ±	+	−	+	+	−	+	−
Dihydrocortisone	375	−	+	−	−	+	−	+	+
11-keto pregnanolone	150	−	+	−	−	+	+	−	−
Compound S (11-desoxycortisone)	450	−	−	−	+	+	−	+	+
Desoxycorticosterone (11, 17-desoxycortisone)	375	−	−	−	+	+	−	−	+

Table VI

STRUCTURAL ASPECTS OF STEROIDS INACTIVE AGAINST MOUSE LYMPHOSARCOMA

Compound	Dose mg/kg/day	11-oxy	Δ^4-3-keto	20-keto	3-OH	17-OH	20-OH	21-OH
Testosterone	500	−	+	−	−	+	−	−
Progesterone	300	−	+	+	−	−	−	−
Δ^5-pregnenolone	50	−	−	+	+	−	−	−
$\Delta^{5,16}$-pregnenolone	187·5	−	−	+	+	−	−	−
Desoxycorticosterone	375	−	+	+	−	−	−	+
21-OH-pregnenolone	375	−	+	+	−	−	−	+
17α OH-progesterone (Δ^4)	250	−	+	+	−	+	−	−
17α OH-progesterone (Δ^1)	250	−	+ Δ^1	+	−	+	−	−
17β OH-progesterone	150	−	+	+	−	+	−	−
17α OH-allopregnanolone (Cmpd L)	200	−	−	+	+	+	−	−
17α, 21-OH-progesterone (Cmpd. S)	375	−	+	+	−	+	−	+
17α-triolone	300	−	+	−	−	+	+	+
17β-triolone	250	−	+	−	−	+	+	+

group has been present in the active steroids, but there has not been an opportunity to test the 20-hydroxy compounds. The 21-hydroxyl group is quantitatively very important, if its presence is not essential.

EFFECT OF CORTISONE
ON LYMPHOSARCOMA IN MICE

CORTISONE 7 14 21 28 | CONTROLS 7 14 21 days

1cm.

[illegible]

Our studies upon the anti-tumour effects have not included a survey of the organ changes. Dr. Woolley has been investigating these effects and has reported that the compounds with an anti-tumour activity in mice depressed the weight

DISCUSSION

SHOPPEE. I was rather surprised to see that in the egg yolk sac experiments Kendall's Compound F showed about 10 times the activity

secondary di-alcohols suggests that they are not metabolized very rapidly.

HADDOW: How reproducible are these different runs?

STOCK. They have been very satisfactorily reproducible, but I don't

process for the human organism. Birds are very different from mammals, and one has to be extremely careful about jumping to conclusions about humans from work on birds. Maybe Dr. Stock can say a few words about the actions of Compounds E, F and A.

STOCK· In our initial experiments with Compound A, it was found active even though somewhat larger amounts were required, and we had the impression that the metabolic side effects were not as bad.

Dr. Karnofsky has been interested to see whether the results in the egg would be carried over to the young chick. He injects the chicks with various doses, usually of the order of 1 mg. per day per chick,

Dr. Burchenal has tested a few of these materials in his mouse leukæmia. He hasn't had the opportunity to test a lot of them because the technique he has used required larger amounts, which were not available.

of the spleen to one-third normal, and the weight of the thymus to one-tenth normal; also they decreased the size of the mesenteric lymph nodes, of the adrenal cortex and of the pituitary. Dr. Money has made similar observations in rats.

Summary

It has been found that cortisone inhibits the development of certain transplantable mouse tumours, osteogenic ones and lymphosarcomas, whereas other tumours are less affected or not at all. Differences in the degree of inhibition of the lymphosarcomas by various steroids suggest relationships of structure to activity. It is believed that the chick embryo and the mouse tumour studies offer methods of screening steroids for biological activity which are both rapid and economical of material. These may be useful for selecting steroids for clinical trial in cancer.

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[illegible]

[illegible] at National Center Sym- 1950.

[illegible] C. P. (1950).

a year.

laboratory which is interested in the production of useful steroids by fungi. They are interested in this test as a possible method of picking up activity.

GROSS: Did you try ACTH too in this test?

demonstrating any possible activity.

tu

STOCK: Yes. For one of our controls we have had animals on a starvation diet where there has been a comparable loss in weight without tumour inhibition. There isn't any question that there is some toxicity. In a few of the experiments nearly all the animals died at the end of the second week from delayed toxicity.

BEGG: I'm a bit uneasy about using this egg embryo test as a screening method. For example, I believe Dr. Karnofsky showed some activity for Compound S in eggs, yet none at all in the tumours.

STOCK: I certainly wouldn't propose this as the ultimate test, but we're not too worried about a few false positives, just as long as there aren't too many of them. It was not expected that the egg test and the tumour test would necessarily yield parallel results.

BEGG: I wondered if we are not seeing examples of the fact that

Stock's studies on pre-natal chicks, we have used the œstrogens as a magnifying glass to bring out differences in tissue growth effects on the genital tract, and, as I showed you yesterday, have found that progesterone has a marked inhibitory effect on œstrogen-induced tissue growth in the genita

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embryo as opposed

same. It may well be that some of them are effective by conversion

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combination of several tests

SHOPPEE: Have you tried to correlate your different degrees of activity, not so much with the actual functional groups present, but simply with the number of oxygen atoms, as an indication of solubility in tissue fluids?

STOCK: We've thought about the possible influence of solubility but there seems to be no correlation.

FOLLEY: *I think that you will find that anhydrohydroxy progesterone* (pregneninolone or ethinyl testosterone), although it contains two oxygen atoms, is one of the most insoluble substances yet tested by the pellet implantation technique. You can't detect any loss in weight in a year.

mixtures. Only a small amount of material is needed. We know one laboratory which is interested in the production of useful steroids by fungi. They are interested in this test as a possible method of picking up activity.

GROSS: Did you try ACTH too in this test?

STOCK: I don't think it's been properly tried out. It hasn't shown much activity. In the animal tests we didn't give it quite frequently enough. We didn't have the opportunity of injecting "around the clock," and I understand from Dr. Woolley that that's essential for demonstrating any possible activity.

THE ANTI-ANDROGENIC CONTROL OF HUMAN CANCER

CHARLES HUGGINS

By anti-androgenic control is meant modification of the internal environment by orchiectomy or administration of œstrogenic compounds.

Anti-androgenic measures are of certain effectiveness in two human tumours: (1) cancer of the prostate; (2) cancer of the male breast. It is likely that this small list will be extended in the future.

As chemotherapeutic agents the sex hormones have one great advantage over all of the other medicines used in the treatment of cancer in that they are not toxic and if they do no good they wreak no great or immediate harm to the patient with cancer. In the treatment of cancer by sex hormones, the aim is to increase the resistance of the patient to his disease.

I shall talk mostly about prostatic cancer because perhaps more is known about this tumour than about any other. The development of the treatment of this lesion by medicines was not empirical; it was deduced from a series of laboratory observations which were carried out, I am afraid, very slowly and at a low intellectual level. The results by good fortune happened to have a sharp focus because the prostate glands are relatively simple, being acted upon, so far as is known, by only two types of hormones, the androgenic and œstrogenic compounds.

Actually, anti-androgenic control of some cancers was devised as a by-product of experiments on dogs in which a quantitative assay technique was instituted. The prostatic secretion is the end product of the male genital hormone complex in which the anterior pituitary, certain hormone

producing cells of the adrenal cortex and testis, and the prostate itself are involved. The prostatic secretion was collected at frequent intervals for rather long periods of time and the effects of substances with endocrine activity on the output of the gland were investigated. In brief, orchiectomy and œstrogen administration caused a decrease of secretion. But androgenic substances initiated, and maintained an augmented prostatic secretion.

It has been said that in any successful experiment there is an element of luck. The good fortune in this work (I do not assert that it was strikingly successful) was the selection of the dog as the subject; this species is the only one other than man to develop spontaneously tumours of the prostate. It was inevitable that sooner or later old dogs with these lesions should be encountered. As a matter of fact, after a short period of vexation because the lesion was interfering with a physiological study, a search was made specially for dogs with this condition.

It was observed that anti-androgenic measures caused a rapid regression of this canine neoplasm whilst androgen increased its size. It was obvious that these effects must be investigated with respect to the malignant and the benign tumours of man, and distinct effects were observed in both conditions, but were more obvious in prostatic cancer.

Now, in science the method of proof is often of greater interest than that which is proven. The effects on disseminated cancer of the prostate were proven by chemical means, namely through a study of the acid and alkaline phosphatases of serum. Acid phosphatase, a component occurring in human prostatic epithelium in rich concentration, had been found, by Gutman and Gutman in 1938, to be increased in the blood serums of certain patients with disseminated prostatic cancer. Now prostatic cancer frequently metastasizes to the bones, in which tissue it flourishes, evoking an overgrowth of the osteoblasts, and Kay had shown in 1929 that when the osteoblasts are stimulated the alkaline phosphatase of serum increased. We could then make a synthesis of these

facts (1941) and study the course of the disease by determining the levels of these enzymes at frequent intervals; of course, acid phosphatase indicated the activity of the malignant cells, while the quantity of alkaline phosphatase indicated the reactivity of non-malignant tissue which was the host of the cancer. In brief, the administration of testosterone increased the activity of the tumour, and anti-androgenic measures caused a decrease of acid phosphatase toward or to normal. Also when the lesion was brought under control there was at first a rise of alkaline phosphatase, indicating a stimulation of osteoblasts (frequently due to a filling in of the bone lesions in the healing process) with a return to normal after some weeks.

The results of anti-androgenic control are of interest from several standpoints. The effect of control is often catastrophic to the tumour and of course beneficial to the patient. There occur an increased appetite and intake of food, and relief of pain. Protein synthesis begins at a rapid rate. The vicious circular chain is broken and the invalid is relieved. Now the striking feature about all of this is its speed; improvement may be detected by chemical means within 24 hours.

The minimal effective dosage of stilbœstrol needed to bring about control has been determined; it is between 0·25 and 0·8 mg. a day of diethyl stilbœstrol administered intramuscularly, thus establishing the fact that control lies within the physiological limits. Diethyl stilbœstrol happened to be the first non-radioactive substance which was found effective against widespread cancer in man.

Statistically, about 95 per cent of patients with prostatic cancer obtain some relief, which is usually considerable. However, a relapse occurs in about 75 per cent of the patients after 6-18 months and these we designate as the failure cases. On the brighter side 20 per cent of patients in our first series are alive and in good health after 10 years, without evidence of disease by chemical or clinical methods. In these people the control certainly has been fairly extensive and moderately long.

In the failure cases certain factors may be discerned. (1) In some of those who relapse there is production of androgen in extra-genital depots which have been identified as the suprarenal glands. (2) Certain of the cancer cells acquire or retain the property of androgen-independence. We conceive of control as the withdrawal, in effect, of androgen from certain cells which require it as an essential nutritive element. Some of the prostatic cancer cells find ways of maintaining themselves in the complete absence of androgen so that escape from control is inevitable.

It should be mentioned that the results from orchiectomy are clinically superior to those obtained from œstrogen administration. In fact, the two methods of androgenic control are in certain ways fundamentally different. After orchiectomy the hypophysis is stimulated and there is much to suggest that the consequent increased adrenal activity is beneficial. Oestrogen in man, on the contrary, depresses the pituitary and this seems to be less beneficial to the patient. Excision of the gonads is the only method known to increase the activity of the pituitary gland for long periods of time.

Since these studies were published, Farrow and Adair have shown that orchiectomy often causes massive regression of the rather uncommon condition of cancer of the male breast. These fine observations of the Memorial Hospital workers have been confirmed in many quarters.

In summary, it may be stated that modification of the internal environment of the host is capable of effecting long continued control of certain widespread cancers of human beings.

DISCUSSION

FOLLEY: You showed a slide of the weight of the dog prostate in which the dog was starved and given testosterone and the prostate grew. You said that you thought it was probably due to waste tissue coming from the catabolism of the body. Have you ever done that experiment in an adrenalectomized dog? As you know, adrenalectomy cuts down tissue catabolism.

HUGGINS: No, we haven't done that. This is a rather widely diversified activity of tissues of the genital tract. The salmon, for example,

in swimming up the river exhausts his proteins but turns out a magni- [illegible]

[illegible] sections look much the same?

HUGGINS: I don't go on sections.

BURROWS: Does the innocent prostate react towards œstrogen in the same way as the malignant ones?

HUGGINS: Yes, it does. It takes quite a lot longer to react. It takes, say, 90 days, whereas a malignant prostate will react in one or two days. [illegible] ptoms because [illegible] still maintain [illegible] oped following [illegible]

[illegible] studied them from the standpoint of acid phosphatase content. I'm not entirely persuaded that these are not metastatic nodules to the breast whose soil has been prepared by œstrogen.

BEGG: This might be a manifestation of multiple tumours. Someone once showed that if you remove the breast cancers from mice who [illegible] and gets a breast cancer that the stilbœstrol necessarily caused the cancer.

HERTZ: It is well established that those who have one primary carcinoma have a greater susceptibility to a second primary than the rest of the population.

HORNING: Do prostatic carcinomas in dogs ever form metastases in the bones?

HUGGINS: They are mostly benign tumours. To the best of my knowledge, spontaneous carcinoma has never been observed in the living animal. Histologically we find that the incidence of carcinoma in these dogs is about 2 per cent, but they have not metastasized.

ADMINISTRATION OF MASSIVE DOSAGE OF ŒSTROGEN TO BREAST AND PROSTATIC CANCER PATIENTS; BLOOD LEVELS ATTAINED

ROY HERTZ, JOHN PAUL YOUNG
and W. W. TULLNER

NUMEROUS clinical reports have established the definite though limited effectiveness of œstrogen therapy in selected cases of prostatic and breast cancer (Huggins, Stevens and Hodges, 1941; Nathanson, 1947).

The transient character of the therapeutic effect of œstrogens in prostatic cancer has been noted by many observers. In breast cancer, both the relative infrequency and the temporary character of œstrogen-induced regressions constitute definite limitations to the practical usefulness of this form of hormone therapy. Nevertheless, the decisive clinical effects obtained with œstrogens in both prostatic and breast cancer command further study aimed at fuller knowledge of the optimum conditions for obtaining such favourable effects.

We have considered the factor of dosage as of paramount importance. By dosage, we do not simply mean the total amount of drug given to the patient, but rather refer to the amount given in relationship to the maintenance of an effective blood level for a stated period of time. In antibiotic therapy, these phases of the dosage problem have been greatly clarified, and the role of massive dosage in the management of such otherwise non-responsive conditions as subacute bacterial endocarditis has been defined. The present report represents a first step in our continuing attempt to determine whether similar principles of dosage and blood level are applicable to the problems of increasing the effectiveness of œstrogen therapy in cancer patients. Moreover, our studies afford data

concerning the tolerance to, and metabolic fate of massive œstrogen dosage.

The diagnosis in all of our cases was initially established by biopsy. Only those patients who were not amenable to any of the established methods of clinical management were selected for these studies. The progress of these patients was followed by constant clinical observation in a research ward. Evidence of alteration in their lesions was recorded by initial and periodic photographs under fixed conditions of illumination and magnification. Toxic reactions to administered œstrogens were continuously assessed by appropriate biochemical studies including such determinations as cephalin flocculation, bromsulphalein retention, serum bilirubin, prothrombin time, eosinophil response to adrenaline, serum calcium and phosphorus, acid and alkaline phosphatase, and frequent urinary and hæmatological analyses. Radiological study of the progress of osseous lesions was carried out when indicated.

We have employed in the major portion of these studies an injectable concentrate of naturally occurring conjugated œstrogens prepared from pregnant mare's urine.* In addition, a more limited number of studies has been done with orally administered ethinyl œstradiol.†

We have recorded previously (Hertz, Tullner, Westfall, Morrow and Emge, 1949) our preliminary studies on a series of breast and prostatic cancer patients. Our current body of data represents observations in fifteen prostatic cancer patients and thirty-six breast cancer patients. Our experience to date includes the administration of 168 intravenous infusions of from 100 mg. to 1,000 mg. equivalents of œstrone sulphate, and 312 subcutaneous infusions of 50–1,000 mg. equivalents of œstrone sulphate.

*The preparation used was "Premarin" (injectable) kindly supplied by Ayerst McKenna & Harrison Ltd., through the courtesy of Drs. G. H. C. McKeown and E. C. Reifenstein, Jr.

†Ethinyl œstradiol was obtained through the Committee on Therapeutic Trials of the A.M.A. and from Ciba & Co., through the courtesy of Dr. E. Oppenheimer.

We have determined the œstrogen blood levels at varied intervals following the administration of a wide range of dosages given subcutaneously, intravenously, and orally. These blood levels are determined by assaying the biological effect of the patient's serum on the weight of the uterus of ovariectomized infantile rats, according to the method of Lauson, Heller, Golden and Sevringhaus (1939). Determinations of blood levels of œstrogens have been made at varying intervals following 87 intravenous infusions, 40 subcutaneous infusions and 21 oral treatments. All assay values are expressed in microgram equivalents of œstrone sulphate per ml. of the patient's serum.

As an example of our quantitative studies of the relationship of dose, injection route, and blood level at varying intervals following treatment, we have selected for presentation here our pooled data obtained from patients given a test dose of 300 mg. of œstrogen. Fig. 1 shows the blood level curve after intravenous infusion of this dose in about 30 minutes in a total volume of 300 ml. A mean blood level of 35 μg. per ml. is obtained and this rapidly falls away with only a very occasional patient showing a significant blood level at the end of 24 hours. The bars indicate the range of variability of the determinations and the number of parentheses indicates the number of determinations made on a given point.

We also studied the effect of giving 300 mg. subcutaneously in 300 ml. volume over about one hour's time. The blood levels attained are of about the same order and these blood levels are maintained for a somewhat longer period of time. Nevertheless, the 24 hour blood level, although somewhat more variable, is only slightly higher than that seen after intravenous infusion.

Oral administration of doses of both crystalline ethinyl œstradiol and of conjugated œstrogens, which approach the limit of gastric tolerance, have afforded much lower blood levels. For example, the highest blood level observed following orally administered conjugated œstrogens was 8 μg. per ml. and this resulted from the ingestion of a total of 3·6 g.

in 29 days. Similarly, after the ingestion of 10 mg. per day for 19 days of ethinyl œstradiol a blood level of only 5 $\mu g.$ per ml. was observed.

Thus, our data afford useful information regarding the relative lack of toxicity of dosages previously considered to

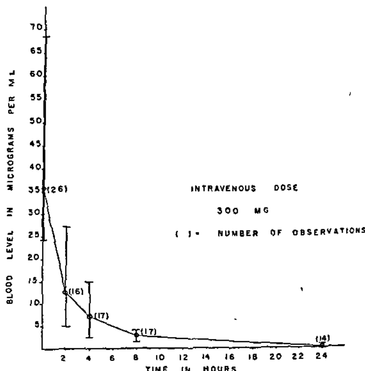


FIG 1. Blood level of œstrogen after intravenous infusion of 300 mg œstrogen in 300 ml. solution over 30 minutes. Pooled data.

exceed the limits of tolerance. In addition, the blood level data indicate the practicability of procuring by parenteral administration blood levels which exceed any that have been obtained by oral administration so far. However, the rapidity with which even the highest blood levels fall away suggested that almost continuous œstrogen administration would be required to maintain a consistently high blood level.

Accordingly, we have made some preliminary studies, employing intravenously placed plastic catheters, of continuous intravenous infusion of large doses of injectable œstrogen in seven patients with advanced cancer of the prostate. This limited experience has indicated that such prolonged infusions may prove a practicable method of maintaining a higher blood level for a longer period of time than is otherwise possible. Fig. 2 shows the record of a twelve hour continuous infusion of a total of 2,330 mg. of œstrogen in the case of a debilitated

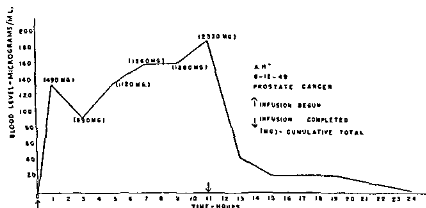


FIG. 2 Blood level of œstrogen in prostatic cancer patient during 12-hour infusion of 2,330 mg. of œstrogen.

prostatic patient. The amount infused at various time intervals is indicated in parentheses and the blood level of œstrogen is graphed against time. Fig. 3 represents our most prolonged infusion to date; it lasted for 72 hours. This involved the administration of 13·3 g. of œstrogen and the almost constant maintenance of a blood level of 400 μg. per ml.

Toxic side effects of such intensive œstrogen administration have proved less severe and less frequent than had been anticipated. Œdema of significant degree was seen in our entire experience in only three patients and this subsided

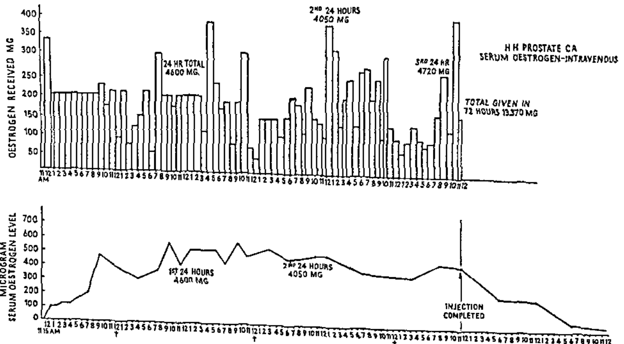


FIG. 3. Blood level of œstrogen in prostatic cancer patient during 72-hour infusion of 13·3 g. œstrogen.

upon interruption of the œstrogen treatment for three to four days and with the use of a salt-free diet. Nausea of varying degree was a recurring complaint of almost all patients in whom a high blood level was maintained for any length of time. In the prostatic cancer patients receiving the more prolonged intravenous infusions, vomiting occurred in all seven but could be controlled in those patients given a liquid diet during the course of the infusion.

In addition, an aged debilitated woman suffering from widely metastasizing breast cancer and severe hypertension died about five minutes after the intravenous infusion of only 400 mg. of œstrogen. The clinical features suggested a cerebrovascular accident. However, a careful autopsy, including complete examination of the brain, showed no apparent basis for her death other than extensive carcinomatosis and hypertensive cardiovascular disease. Also, one of the prostatic cancer patients, 62 years of age, who had been given 40 hours continuous intravenous infusion of injectable œstrogen abruptly presented a picture of peripheral vascular collapse necessitating discontinuation of the infusion. His pulse became rapid and thready and his blood pressure was unobtainable. His abdomen was noted to be distended and rigid and epigastric tenderness was elicited. Since he had had in the past a perforated peptic ulcer requiring surgical intervention, it was considered that he then was again suffering from perforation. During the ensuing two days, he responded quite well to transfusions and other supportive measures and a blood urea nitrogen of 44 mg. per cent was reported. On the third day, he became comatose and irrational and died. Autopsy revealed an intact gastro-intestinal tract. The kidneys showed a diffuse parenchymatous swelling associated with lower urinary tract obstruction due to extensive carcinomatous invasion. It was considered that death had occurred from uræmia.

Hence, despite careful autopsy examination in these two cases, it remains difficult to determine the role of the œstrogen as a toxic factor. However, in view of the lack of major

toxicity in numerous patients much more intensively treated than these two, it is reasonable to consider that the fatal course in these two patients can be more readily attributed to other phases of their advanced disease-state.

Laboratory studies referred to above have indicated no evidence of hepatic, renal or hæmatological damage in any of our cases.

Our data so far are admittedly of more pharmacological than therapeutic interest. We have seen significant therapeutic effect from massive œstrogen dosage in five prostatic cancer patients and in five breast cancer patients. In the prostatic cancer patients these favourable effects included: (1) a rapid reduction in size of the palpable prostatic mass; (2) relief of severe bone pain with an associated abrupt drop in serum acid phosphatase levels; (3) general clinical improvement as regards weight, appetite and feeling of well being. In the breast cancer cases, these effects have included: (1) prompt and marked regression in the visible breast lesion; (2) marked suppression of pleural effusion; (3) decisive relief of pain from bone metastases; (4) reduction in size of metastatic lymph nodes; and (5) general clinical rehabilitation of the patient.

Fig. 4 shows the treatment record over a period of 49 days of E.B., a 75 year old white female who had received no prior treatment for her breast cancer. One thousand milligrams of œstrogen subcutaneously were given almost daily throughout the entire period. Fig. 5 shows the initial appearance of the breast lesion in contrast to its appearance when treatment was discontinued seven weeks later. The gross regression which was accompanied by a marked rehabilitation of the patient is apparent. Fig. 6 shows the course of this lesion in greater detail at about two week intervals during therapy. Such rapid and decisive clinical effects as this have stimulated our interest in further evaluating this intensive form of hormone therapy.

In conclusion it should be emphasized that this report is offered to indicate only some initial progress toward



A



B

FIG. 5. Patient of Fig. 4 before treatment (A) and after seven weeks treatment (B).

[To face page 164

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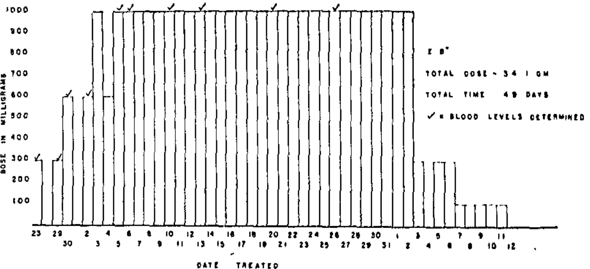


FIG. 4. Œstrogen treatment record of breast cancer patient, given 34.1 g. œstrogen over 40 days.









Fig 6 Lesion of patient in Figs 4 and 5 at two-week intervals during therapy.

better.

I'm not at all persuaded that tremendous doses of these things are what is needed. I think, from my limited experience, that minute

Hertz: In the two patients who died in the course of these studies the adrenal was not unusually large; they had extensive carcinomatosis. And we have also followed very carefully the eosinophil reactivity to an administered dose of adrenaline of the patients during the course

tremendous amounts of chromogenic material.

Dobriner: There are a number of reports that in mice, rats and rabbits the adrenals increase in size after treatment with œstrogens, but this may not always involve an increase in adrenal function.

allow a cancer cell to adjust to the œstrogen by teasing it with small dosages we may be able to suppress it more effectively

Haddow: Our experience at the London Cancer Hospital in both

establishing a quantitative base line in œstrogen therapy of cancer so that more effective forms of hormone therapy may ultimately be evolved. Our data are to be interpreted as indicating: (*a*) the feasibility of massive parenteral œstrogen administration; (*b*) the quantitative limits of the blood levels attained; and (*c*) sufficient clinical therapeutic effect to justify *further exploration of massive œstrogen therapy under* controlled conditions of clinical study.

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DISCUSSION

HERTZ: I failed to mention that we have used progesterone effectively and have seen no toxic side effects, except in one patient who was sensitive to the oil vehicle and showed an allergic reaction, so that we felt that progesterone was sufficiently inert to allow us to go up to very high dosages. Other steroids will have to be approached much more cautiously.

STOCK: Although progesterone showed marked activity in our tests, I would not have expected it to be effective necessarily in clinical trials, because we would have anticipated that there would be false positives. On the other hand, we may be missing activities in some compounds by testing them against only one type of tumour, and we

we
have

lear
hat

the clearing was of renal origin?

HERTZ: Yes. We haven't completed our studies, but we know that *30–50 per cent of the administered œstrogen given in such massive* dosage is found in the urine in the next 26–36 hours. However, even when such an enormous dosage is given, we still can't account for it all, and perhaps there is an additional inactivating mechanism involving the inactivating capacity of other tissues. There may also be loss from the bowel, and we are intending to study that.

HUGGINS: At one time we thought it would be a good idea to synthesize stilbœstrol phosphate because it is a water-soluble ester. It

one because of the small amount of sulphatase, other esters might be better.

I'm not at all persuaded that tremendous doses of these things are what is needed. I think, from my limited experience, that minute

Hertz. In the two patients who died in the course of these studies the adrenal was not unusually large; they had extensive carcinomatosis. And we have also followed very carefully the eosinophil reactivity to

initial eosinophil level, which would be low if the adrenal were activated. The biochemical studies on the urine are limited by the presence of

Hertz's results

The only similar investigation I know of is one carried out by Dodds. It was not published, although I think there is a reference to it in the annual reports of the British Empire Cancer

Campaign two or three years ago. He administered amounts of the order of grams of one or more water-soluble derivatives. Perhaps Boyland could remember which ones?

... found stilboestrol sulphate and hexœstrol ... there's ... jection ... a con- ... would have to convert it into a water-soluble derivative before excretion, and the blood concentration would be maintained. In general, as *Albert has stressed, increasing the solubility of a substance very* frequently reduces the biological activity.

HERTZ: *We have studied the blood levels in patients who were carry-* ... of both stilboestrol and steroid types of ... Our working hypothesis ... we can attain a blood ... get a therapeutic effect ... whether or not it is biologically active, the vaginal smears in these patients show very definite cornification.

BEGG: Did you show progesterone inhibition of œstrogen-induced tissue growth in the mammal?

HERTZ: In the mammal in moderately low dosage progesterone is *complementary to the growth-stimulating action of œstrogen*, in terms of weight increment in the genital tract. However in some of our studies, when we used large dosages, 3–4 mg. a day, we found the same ...

... rogesterone did you notice any ... Trentin has recently observed ... mice that have received, over a ... *of progesterone a day.* It is a rather significant increase in weight, more than you can get in these animals with testosterone. He hasn't had it analysed for fat or protein yet.

I think that Dr. Folley mentioned that the mammary glands, ... grew disproportionately, and there is ... the monkey's ... *fect for* a long ... far. I think it ... ies or organ to organ.

... Or from benign to malignant?

... ht very closely and have ... *weight due to improve-* ... we have been concerned ... ne as seen in pregnancy ... reflected to some extent ... For that reason we

studied the histidine excretion in these patients, and found that progesterone, even in these large doses, will not give the histidinuria, whereas cortisone will. Pregnanediol excretion, as done by Sommerville and Marrian's method, has been within the expected range of normal recovery, indicating that we're not dealing with any great abnormality in steroid metabolism in these patients.

Burchenal: I would like to support Dr. Hertz's theory about the value of large dosage. We've certainly seen that with cortisone in acute leukæmia. A child given 50 mg. a day, which will work with some children, may go on for two months with no improvement at all; then you increase the dose to 100 mg. a day and even with that you get no improvement; but if you get it up to 200 mg. a day, you may see a rapid betterment of the general condition and a reversion of the bone marrow to normal.

Gross: Can you observe the same kind of regression with testosterone in cervical cancer?

Hertz: We have not studied the effect on cervical cancer.

Dobriner: I think we have to correct our thinking about dosage. The amounts one used to call "veterinarian" are produced by humans. For therapeutic application, I think one has to see what an *adequate* amount is. It is possible that there is a maximum dosage, and an increase doesn't help. We have to pay much more attention to the blood level. One reason that Compound E, for instance, is given in four or eight doses per day is that one has to attain a certain blood level. The same thing may be true for the œstrogens and testosterone. I think that Dr. Hertz is on the right track when he uses such a high dose. If we could release the hormone steadily and imitate the action of the glands, I think our therapy would be very much more successful.

Haddow: At the Cancer Hospital we had almost stopped considering any more the use of large amounts of hormones, but Dr. Hertz's paper makes us think about them again. I am particularly impressed by the dramatic regression with progesterone withdrawal

THE MODIFICATION OF TUMOUR-HOST RELATIONS BY STEROID HORMONES

R. W. BEGG

I. Systemic Effects of Tumours in Rats

SYSTEMIC effects of tumours are regarded as the changes produced in tissues of the host which are remote from the tumour, and in which no evidence of metastatic malignant cells is found. They may be anatomical, of a nature described by Parsons (1947), or of the type of biochemical change reviewed by Greenstein (1947).

It has been suggested on histological grounds that the clinical state of malignant cachexia is due to hypofunction of the adrenal (Dalton, 1944) and this view may be supported by certain clinical studies (Potor *et al.*, 1948; Reifenstein *et al.*, 1948; Young *et al.*, 1948). In view of the number of metabolic factors known to be influenced by the adrenal cortex (Thorn and Forsham, 1949), the possibility that some systemic effects could be explained by a primary action on the pituitary-adrenal system was investigated.

Liver catalase activity was chosen for the study as a well established systemic effect (Adams, 1950; Appleman, Skavinski and Stein, 1950; Dounce and Shanewise, 1950; Greenstein, Jenrette and White, 1941); adrenalectomy leads to a diminished activity of liver catalase (Begg and Reynolds, 1950). Hæmoglobin and liver catalase have a common prosthetic group (Theorell, 1947) and anæmia has been related to tumour growth (Taylor and Pollack, 1942) and to the adrenal cortex (White and Dougherty, 1945).

The enlarged adrenal, low in ascorbic acid and cholesterol, which has been described in the tumour-bearing animal (Savard, 1948; Haven, Bloor and Randall, 1949) would be compatible with exhaustive hypofunction of the adrenal

cortex (Sayers and Sayers, 1948), particularly in conjunction with the diminished liver glycogen deposition (Young, Kensler, Seki and Homburger, 1947) and lymph node hypertrophy (Homburger, 1948) which have been demonstrated in tumour-bearing mice.

It was decided to study these multiple systemic effects in a single animal at different stages of tumour growth, and the rat was chosen to provide the required amount of tissue. Thymus weight was followed as an example of lymph tissue, for it was assumed to react to experimental procedures in a manner similar to lymph nodes (White, 1947).

Young male Sprague-Dawley-Holtzman rats were maintained on a diet of Purina fox chow and tap water in a room maintained between 72 and 78°F. The rats were bred in the laboratory or supplied by the Holtzman Rat Company, the latter being adapted to the animal room for at least ten days before use. Bilateral grafting was done aseptically when the rats were approximately six weeks of age, a tumour suspension being used. The Walker 256 carcinoma was grafted either subcutaneously or intramuscularly, but only the latter method was used for the Jensen sarcoma. Normal male rats served as controls and were killed at the same time as tumour-bearing rats. Twenty hours before sacrifice the rats were placed in clean cages without food but with water supplied *ad libitum*. At this time hæmoglobin was determined on tail blood (Evelyn, 1936).

The rats were injected intraperitoneally with sodium pentobarbital in warm normal saline at a dose level of 5 mg. per 100 g. body weight. Under anæsthesia the abdomen was opened, the right adrenal transferred to formol-saline and the left placed in a dish of ice-cold normal saline. A second operator removed a piece of liver for glycogen determination (Good, Kramer and Somogyi, 1933). This was rinsed in ice-cold saline, blotted dry, weighed and introduced with minimum delay into a tube of hot 30 per cent potassium hydroxide. Meanwhile the left adrenal had been freed of adherent fat, blotted dry, sectioned with a razor blade and weighed on a

torsion balance while wrapped in cellophane. One piece was introduced into trichloracetic acid for estimation of ascorbic acid (Roe and Kuether, 1943). The other half was homogenized with a loose pestle in a tube containing acetone, and an equal amount of absolute alcohol added for the extraction and determination of cholesterol (Sperry, 1938) The remainder of the liver was ground and extracted for the estimation of liver catalase activity (Greenstein, 1942) and the thymus weighed on a torsion balance. After 48 hours fixation the adrenals were washed in running tap water for an hour, sectioned on a freezing microtome and stained with Sudan IV (Conn, 1946).

The body weights recorded are those prior to sacrifice and include both tumour and carcass weight. The tumours were measured in two diameters and are presented as the mean diameter of both tumours. Tumours were not weighed in all cases, but sufficient data are available to state that in the 20 mm. group they formed approximately 5 per cent of the body weight, in the 30 mm. group, 15 per cent and in the 40 mm. group 30 per cent of the body weight. With the subcutaneous grafts the tumours attained 10, 20, 30 and 40 mm. diameters in one, two, three, and four weeks respectively. The intramuscular grafts grew at a somewhat faster rate.

Values of P in the t test of Fisher are regarded as significant at the 0·05 level and highly significant at 0·01 (Snedecor, 1946).

Results

The Walker and Jensen tumours gave comparable systemic effects and the results obtained from rats bearing these tumours have been combined.

The mean body weights of the different groups are given in Table I. There is no significant difference between the mean weights of the groups, and the adrenal and thymus weights are thus regarded as subject to valid comparison between groups.

Thymus and adrenal weights are presented in Table II. There is a progressive increase in adrenal weight and a fall in

thymus weight after the tumours have attained a size of 20 mm.

The results of the estimation of hæmoglobin and liver catalase activity are tabulated in Table III. Hæmoglobin

Table I

BODY WEIGHT OF CONTROL AND TUMOUR-BEARING RATS

Tumour size	*Body weight (g)*
Control	170±4(7)
10 mm	160±7(16)
20 mm.	161±7(10)
30 mm.	173±11(10)
40 mm	182±12(10)

±Standard error of the mean.
Number of observations in brackets.

Table II

EFFECT OF TUMOURS ON ADRENAL AND THYMUS WEIGHT

Tumour size	*Adrenal weight (mg)*	*Thymus weight (mg)*
Control	15·4±0 8(7)	387±2 3(7)
10 mm	17·5±0 4(16)†	420±85(10)
20 mm.	18 8±0·3(10)*	324±45(12)
30 mm	21·3±2 0(10)†	288±86(5)
40 mm.	30 2±4·0(9)*	156±29(8)*

±Standard error of the mean.
Number of observations in brackets.
*P< 0 01 in *t* test
†P< 0 05 in *t* test

Table III

EFFECT OF TUMOURS ON HÆMOGLOBIN AND LIVER CATALASE ACTIVITY

Tumour size	*Hæmoglobin (g/100 ml)*	*Liver Catalase Activity ($K \times 10^4$ at 0 1 mg N/ml)*
Control	15 4±0 3(7)	1570±74(6)
10 mm.	16 2±0 8(16)	2079±132(16)*
20 mm.	11 8±1 2(10)†	1596±119(10)
30 mm.	10 7±1 1(10)*	1332±125(10)
40 mm.	8 6±0 5(10)*	753±81(10)*

±Standard error of the mean.
Number of observations in brackets
*P< 0 01 in *t* test
†P< 0 05 in *t* test.

falls progressively from the 20 mm. tumour to attain a pre-agonal value of approximately 50 per cent. The loss of liver catalase activity is of the same order. The increase in catalase activity in rats bearing small tumours has been reported (Greenstein *et al.*, 1941) and is significant in this series.

Table IV

EFFECT OF TUMOURS ON ADRENAL CHOLESTEROL AND ADRENAL ASCORBIC ACID

Tumour size	*Adrenal cholesterol (mg /100 mg)*	*Adrenal ascorbic acid (mg /100 mg)*
Control	4·68±0·52(7)	0·413±0·012(7)
10 mm.	4·16±0·38(16)	0·372±0·012(15)†
20 mm.	3·79±0·52(10)	0·324±0·017(10)*
30 mm.	3·25±0·45(9)	0·314±0·022(9)*
40 mm.	2·97±0·44(10)†	0·238±0·027(9)*

±Standard error of the mean
Number of observations in brackets
*P< 0·01 in *t* test.
†P< 0·05 in *t* test

In Table IV *the effects on adrenal cholesterol* and ascorbic acid have been recorded. The values for cholesterol and ascorbic acid fall to 60 per cent of the control at the 40 mm. stage.

Histological examination revealed a loss of sudanophilia from the adrenals of the tumour bearing rats. The loss of sudanophilia paralleled the loss of cholesterol from the adrenal.

Discussion

The Walker 256 carcinoma at an early stage of growth reduces the food intake of the host, and as the tumour increases in size the carcass loses weight (Mider, Tesluk and Morton, 1948). The suggestion has been made that this may be a factor in the loss of liver catalase (Dounce and Shanewise, 1950). Experiments have been reported which indicate that in the forcibly fed tumour-bearing rat no loss of carcass weight occurs, but systemic effects are present (Begg, 1950) Thus, failure to record food consumption in this study does not render the results invalid.

The present experiments are not in agreement with the statement that tumours forming 5 per cent of the body weight cause a diminution in catalase activity (Greenstein, 1947) but do agree with observations that half the liver catalase activity is lost from animals bearing tumours of 15 to 30 per cent of the body weight (Dounce and Shanewise, 1950). The catalase effect appears to be biphasic, as has been reported by Greenstein (Greenstein *et al.*, 1941) in rats but not noted by Adams (1950), who found an asymptotic relation between tumour weight and percentage of inhibition of catalase, in mice.

The earliest significant effect produced by a tumour in this investigation is the hypertrophy and loss of ascorbic acid from the adrenal at the 10 mm. stage, followed by anæmia at the 20 mm. stage. That the degree of anæmia is a consistent and reproducible finding in tumour-bearing rats is suggested by the fact that comparable groups of rats bearing the Walker carcinoma yielded hæmoglobin values of $9 \cdot 34 \pm 0 \cdot 49$, $8 \cdot 50 \pm 0 \cdot 40$ and $8 \cdot 03 \pm 0 \cdot 44$ g./100 ml. at intervals of six months.

It was suggested that diminished liver catalase activity and anæmia might be associated with hypofunction of the adrenal cortex in the tumour-bearing rat. Anæmia has been produced by adrenalectomy in mice (White and Dougherty, 1945), and it has been demonstrated that adrenalectomy lowers liver catalase activity in the rat (Begg and Reynolds, 1950). But the diminution in catalase activity in the rat from which the adrenals are removed is only half as great as in the tumour-bearing animal Thus it is unlikely that hypofunction of the adrenal cortex is a major factor in the production of anæmia and loss of liver catalase activity in the tumour-bearing rat.

The observed involution of the thymus in the tumour-bearing rat might be regarded as indicative of hyperfunction of the adrenal cortex (Selye, 1936) or inanition (Reinhardt, 1943). Extrapolation to another species should be done with caution, but thymus involution and lymph node hyperplasia have been observed in tumour-bearing mice (Homburger, 1948; Savard, 1948) and appear to be independent of the

pituitary (Savàrd and Homburger, 1949). This throws some doubt on the interpretation of the present data, and further studies should be done to investigate the response of lymph nodes as well as the thymus to the presence of a tumour in the rat, and the role of the adrenal (White and Dougherty, 1947).

The results obtained from the study of adrenal weight, adrenal ascorbic acid and cholesterol, and sudanophilia, might be interpreted as following the pattern of the stress reaction as exemplified by the Type III adrenal response of Sayers (Sayers and Sayers, 1948), on an extended time scale. This could be the result of an initial stimulation of the adrenal cortex being maintained and leading to eventual cortical exhaustion.

The progressive fall in adrenal cholesterol and ascorbic acid would suggest that the host was approaching the stage of adrenal cortical failure (Sayers and Sayers, 1948). In the absence of the determination of peripheral effects known to be influenced by the secretions of the adrenal cortex, these changes in the adrenal are difficult to interpret. The determination of liver glycogen in rats starved for sixteen hours gives such low results, and the data are so variable, that these experimental findings have not been presented. A plot of mean percentage change does indicate an initial increased storage of glycogen, with a diminution in the amount of liver glycogen in the terminal state. This problem should be studied with the use of intraperitoneal glucose (Young *et al.*, 1947).

It is probable that a state of adrenal cortical deficiency does occur in the tumour-bearing animal in the terminal stages of cancer, but further experimental substantiation is required. All of the systemic effects cannot be explained on the basis of this deficiency, and some other mechanism must be responsible for many factors known to be altered in the tumour-bearing host.

As the result of the present and related investigations it is necessary to explain how the presence of a tumour at a remote site brings about a stimulation of the adrenal cortex, a

diminution in the amount and activity of hæmoproteins and an involution of the thymus. Neither hormonal nor nutritional explanations seem to be adequate.

In his original studies on liver catalase in tumour-bearing rats Greenstein suggested that "the effect of the transplanted tumor on the liver catalase is elicited by a toxic substance produced in the tumor and carried by the blood to the liver" (Greenstein *et al.*, 1941). A recent abstract reports that such a substance can be extracted from a tumour (Greenfield and Meister, 1950). Adams (1950) favours the release of some substance from a tumour as an explanation of the diminished liver catalase activity of mice. A consideration of the systemic effects of tumours may lead to the revival of the concept of a cancer toxin and stimulate further research along this line.

A claim for specificity in relation to malignant tumours cannot be made for the observed systemic effects, and no explanation is available to account for these characteristic effects in tissues remote from a tumour. At the same time they are manifestations of the profound metabolic changes produced in the host, and as such it is felt that they play an important part in the fatal outcome of cancer.

II. Steroid Hormones and Tumour-Host Relations

It has been demonstrated that testosterone propionate in oil causes an increase in hæmoglobin and liver catalase activity in the normal rat, but that the administration of the steroid in large doses could not overcome the characteristic anæmia and loss of liver catalase activity in the tumour-bearing rat (Begg, 1949). It was of interest to see if these effects might be moderated by the administration of a more potent androgen.

The use of pellets of testosterone propionate provided another approach to the problem. Implanted in the subcutaneous tissues these would give a slow steady absorption of the hormone which might be of greater effect than daily injections in oil.

A pattern of the systemic effects of tumours has been described and evidence presented to suggest that rats in the terminal stage of cancer may be in a state of adrenal cortical insufficiency (Part I). If this reasoning were valid it might be anticipated that an exogenous supply of hormones of the adrenal cortex would modify the response of the host to the tumour.

Methods

A group of young male Sprague-Dawley-Holtzman rats bearing intramuscular grafts of the Walker 256 carcinoma in both thighs was used as test animals. On the ninth day of growth, when the tumours had attained a diameter of 10 mm., injections were begun or pellets implanted. The animals were maintained on Purina fox chow and tap water in a room controlled at 72–78°F.

Testosterone cyclopentylpropionate* (TCP) was given in two 5·0 mg. doses, one on the ninth and one on the fourteenth day of tumour growth. Lipo-Adrenal Cortex† (LAC) was administered in daily injections of 0·5 ml. increasing to 1·5 ml., different groups receiving 240, 360 and 420 rat units respectively as a total dose. Control animals were given appropriate amounts of cottonseed oil. Pellets of testosterone propionate‡ (TPP) were implanted over the scapular region, five pellets per rat, each placed at a discrete site. At autopsy the pellets were removed, dried and reweighed for the determination of total absorption.

Hæmoglobin was estimated on tail blood on the afternoon preceding sacrifice (on the twentieth day of tumour growth),

*Testosterone cyclopentylpropionate was provided by the Upjohn Company through the kindness of Dr. H F Hailman. The Upjohn Company report that this compound has a more potent and prolonged androgenic activity than testosterone propionate

[illegible] vegetable [illegible] milli- [illegible] pound

‡Pellets of testosterone propionate were provided by the courtesy of Dr B. L Frank of the Ciba Pharmaceutical Company (Canada). They weighed approximately 15 mg each and were known to give an absorption of about 0 2 mg per pellet per day

control rats being killed at the same time. The two androgen groups were given access to food and water till the end of the experiment, but the LAC group were starved for sixteen hours and given 400 mg. glucose intraperitoneally two hours before sacrifice.

Tissues were removed under nembutal anæsthesia and analysed by methods described in Part I.

Tumours were measured in two diameters of each tumour and are expressed as the mean of the four diameters. In these experiments the tumour weight corresponds to approximately 25 per cent of the body weight.

Results

Testosterone Cyclopentylpropionate

Table V indicates that the only significant difference between the control and treated groups is increased thymus atrophy in the rats given TCP. The mean adrenal weight is

Table V

EFFECT OF TESTOSTERONE CYCLOPENTYLPROPIONATE ON TUMOUR BEARING RATS

	Control	*TCP*	P^*
Body weight, g	245 1±7 3(10)	242 8±8 4(9)	>0 05
Tumour diameter, mm.	39 0±1 0(10)	39 1±1 3(11)	>0·05
Adrenal weight, mg	28 5±0 7(8)	26 5±1 2(11)	>0 05
Thymus weight, mg	103 4±20 7(8)	105 2±14 0(11)	<0 01
Hæmoglobin, g/100 ml	8 03±0 44(10)	7 67±0 72(11)	>0 05
Liver catalase, K×10⁴	1964±142(10)	2053±213(11)	>0 05
Adrenal cholesterol, mg /100 mg.	2 24±0 28(8)	1 73±0 20(11)	>0 05
Adrenal ascorbic acid mg /100 mg	0 315±0 029(8)	0 315±0 017(11)	>0 05

Number of observations in brackets
± Standard error of the mean
*Probability in *t* test $< 0\ 05$ = significant
$< 0\ 01$ = highly significant

smaller in the treated group, but the variation considerable; a similar response is noted in adrenal cholesterol. The failure to influence hæmoglobin and liver catalase activity is evident.

Testosterone Propionate Pellets

As indicated in Table VI there is no apparent effect on

Table VI

EFFECT OF TESTOSTERONE PROPIONATE PELLETS ON TUMOUR BEARING RATS

	Control	*Pellets*	P*
Body weight, g.	245 1±7 3(10)	230·2±3 2(11)	>0 05
Tumour diameter, mm.	39 0±1 0(10)	38 6±1·0(11)	>0 05
Adrenal weight, mg.	28 5±0 7(8)	25 5±0·9(11)	<0 05
Thymus weight, mg.	193·4±20 7(8)	50 8±4 6(11)	<0 01
Hæmoglobin, g/100 ml.	8 03±0 44(10)	8 39±0 70(11)	>0 05
Liver catalase, K×10⁴	1964±142(10)	2309±118(11)	>0 05
Adrenal cholesterol, mg /100 mg.	2·24±0 28(8)	1 09±0·23(10)	<0·01
Adrenal ascorbic acid, mg /100 mg	0 315±0 029(7)	0 306±0 018(11)	>0 05

Number of observations in brackets.
±Standard error of the mean.
*Probability in t test. < 0 05 = significant.
< 0 01 = highly significant.

adrenal ascorbic acid but adrenal weight and cholesterol are both significantly lower in the treated group. The pellet treated rats showed a marked involution of the thymus but there was no significant effect on liver catalase activity or hæmoglobin.

The average absorption of testosterone propionate from the pellets was 0·99 mg. per rat per day.

Lipo-Adrenal Cortex

An examination of Table VII demonstrates that the injection of adrenal cortical extract produced significant changes only in thymus weight, hæmoglobin and body weight.

The mean weight of the two groups was the same at the beginning of the injections. It is difficult to interpret the decreased gain in body weight in the treated group as the animals were not tube fed, and the reduction in weight gain may be a reflection of a decreased food consumption. The

characteristic loss of sudanophilia in the adrenals of tumour-bearing rats was not so marked in the treated as in the control group.

Table VII

SPRAGUE-DAWLEY RATS BEARING WALKER 256 CARCINOMA

	Control	*Lipo-adrenal cortex*	P*
Body weight, g.	323·4±7·2(11)	299·3±5·1(12)	<0·07
Tumour diameter, mm.	46·3±2·0(12)	45·8±1·2(12)	>0·05
Adrenal weight, mg.	29·4±1·6(11)	27·4±1·5(10)	>0·05
Thymus weight, mg	207·6±17·6(11)	88·0±6·0(10)	<0·01
Hæmoglobin, g/100 ml.	8·5±0·4(11)	10·4±0·5(12)	<0·01
Liver catalase, K×10⁴‡	933±94(11)	759±123(10)	>0·05
Adrenal cholesterol, mg/100 mg.	2·64±0·30(11)	3·28±0·44(10)	>0·05
Adrenal ascorbic acid, mg/100 mg.	0·314±0·016(11)	0·291±0·015(10)	>0·05
Liver glycogen, mg/100 mg.†	0·44±0·05(11)	0·45±0·06(10)	>0·05

Number of observations in brackets.
± Standard error of the mean
*Probability in t test: < 0·05 = significant
< 0·01 = highly significant.
†Expressed as glucose, two hours after 400 mg glucose intraperitoneally
‡Determined on a liver extract prepared by grinding with sand in a mortar. This procedure gives lower results than an extract prepared in a Waring blender, as in the androgen experiments, but the ratio of activity in the livers of control and tumour bearing rats is the same in both methods

It will be noted that in no instance was tumour growth inhibited, and histological examination of the tumours from the treated animals did not show any variation from the control group.

Discussion

The magnitude of a systemic effect is related to the size of the tumour (Part I). For this reason it is essential in any attempt to modify systemic effects that there be no difference in the growth-rate and size of the tumours in the control and treated groups.

Steroid hormones have been shown to affect the growth of mammary tumours in the human, and the response of the host to the tumour (Therapeutic Trials Committee, 1949). In the rat bearing the Walker 256 carcinoma there is no effect

on the tumour and only a slight influence on tumour-host relations. Haddow (1950) has directed attention to this discrepancy of the effect of chemotherapeutic agents in the laboratory and in the clinic.

The experimental demonstration that TCP is a more potent androgen than testosterone propionate does not mean that it would necessarily have a greater effect on hæmoglobin and liver catalase activity. Studies on the metabolic effects of the androgenic hormones do not permit yet of a correlation between action on enzyme systems and protein synthesis and the effects on the seminal vesicles and prostate of the castrated immature rat. Kochakian (1947) has demonstrated that some steroids with a very weak or absent androgenic activity may have a moderate influence on alkaline phosphatase and thymus involution.

The thymus involution in tumour-bearing rats implanted with pellets of testosterone propionate is the greatest that has been observed. The gland is reduced in most instances to a small thread-like structure. This marked involution is produced by the absorption of 1 mg. per day from the pellets, and exceeds that resulting from the injection of 2 mg. a day of the same steroid in oil.

Adrenal hypertrophy was reduced in degree by pellets of testosterone propionate, and it might be inferred that this was the result of inhibition of release of ACTH from the pituitary (Bartter, Forbes, Jefferies, Carroll and Albright, 1949; Faloon, Owens, Broughton and Gorham, 1950). If this were true, an increase in adrenal ascorbic acid might have been expected but was not demonstrated.

Unpublished experiments indicate that pellets of testosterone propionate do not alter the urinary excretion of nitrogen, sodium and chloride in tumour-bearing rats.

The extract of adrenal cortex was able to reduce the degree of anæmia but had no influence on the loss of liver catalase activity. It has been reported that an aqueous extract of the adrenal increases hæmoglobin in the rat (Yoffey and Baxter, 1946).

There appears to be some variation in the response of tumours to steroids of the adrenal cortex (Sugiura, Stock, Dobriner and Rhoads, 1950), but an inhibition of growth of the Walker 256 carcinoma has been reported (Ingle, Prestrud and Rice, 1950). This occurs in the presence of an inhibition of body growth more marked than in this series.

The failure to affect the adrenal by the LAC may be in the design of the experiment. The mean weight of the adrenal in the treated group is smaller and the cholesterol content higher than in the control. If the series were expanded to include larger numbers of animals the differences might become significant. Such a possibility is supported by the fact that LAC did have a sparing action on the loss of adrenal sudanophilia, which has been correlated with cholesterol (Sayers, Sayers, Fry, White and Long, 1944).

The failure to produce anticipated effects may be more fundamental, and may be due to an inadequate dosage of LAC. It has been estimated that the daily output of the adrenal of the rat is the equivalent of twenty-five millilitres of an aqueous extract of the adrenal cortex, based on the amount required to permit normal performance of the work test in the adrenalectomized rat (Ingle and Nezamis, 1948).

Summary

Tumour-bearing rats exhibit enlargement of the adrenal, with loss of ascorbic acid and cholesterol, atrophy of the thymus, diminution in liver catalase activity and progressive anæmia.

The thesis that rats bearing large tumours are in a state of hypofunction of the adrenal cortex requires further substantiation, and in any event such a state would not explain the observed systemic effects.

Pellets of testosterone propionate reduce adrenal hypertrophy in the tumour-bearing rat, and an extract of the adrenal cortex diminishes the degree of anæmia. No effect was noted on the growth of the Walker 256 carcinoma.

The author is indebted to Dr. H. P. Rusch of the McArdle Memorial Laboratory for donor rats carrying the Jensen sarcoma and the Walker

256 carcinoma, and to Mr. T. E. Dickinson, B.Sc., and Mr. D. G. Withers for technical assistance. The work was begun during the tenure of a British Council Scholarship at the Sir William Dunn School of Pathology, University of Oxford.

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DISCUSSION

BOYLAND: It is quite clear that the changes in liver catalase and hæmoglobin do not go together. Injection of suitable extracts of [illegible]

shortage of liver catalase [illegible]

[illegible]

arginase in tumour-bearing animals.

BEGG: Greenstein supported that view.

HADDOW: I am always a little anxious that effects of the kind D Begg described are not secondary. We may have minor degrees infection which do not substantially hold up the tumour but mayl responsible for some of these effects. Have you any view on the possibilities?

BEGG: I think it's very difficult. We have tried to limit our resul to cases where the tumours were the same size and of standard micro scopic appearance. If you're studying tumour-host relations, yo must keep to a standard size, otherwise you would get a greater or le amount of catalase simply from a larger or smaller tumour.

HERTZ: I think this matter of the mystery regarding the mechanism of death of cancer patients has been very much over-emphasized [illegible]

eventually die, in how many would you expect to get hæmorrhage intestinal obstruction and so on?

HERTZ: The experimental situation is different from the clinical.

BEGG: Clinically, although a large proportion of patients have ha some metastases to the lung and some to the liver, there are many people who have much greater destruction from old tuberculosis an old cirrhosis and live quite happily.

PART IV

CLINICAL AND METABOLIC EFFECTS OF ACTH AND CORTISONE IN NEOPLASTIC DISEASES

STEROID HORMONES AND CANCER

KONRAD DOBRINER

THE relation of steroid hormones to the cancer problem has been investigated for more than 40 years in both animal and clinical studies. The results have been reviewed at intervals by a number of authors (Lacassagne, 1939; Allen, 1940; Gardner, 1947; Nathanson, 1947; Lipschutz, 1950). Even before knowledge of the structure and the physiological action of the steroid hormones was available, keen observations by Lathrop and Loeb as early as 1916 indicated that gonadal function was concerned in the ætiology of cancer. These workers reported that early gonadectomy caused a marked reduction in the incidence of spontaneous mammary cancer in mice. After the isolation of œstrone by Doisy and Butenandt in 1929, Lacassagne (1932, 1939) in 1932 found that administration of pure female sex hormone produced mammary cancer in male mice of high cancer strains. Since then, in a great number of investigations, the role of the sex hormones in the ætiology of cancer has been explored by the removal of the gonads as well as by the administration of male and female sex hormones Gardner (1947) in his review of the steroid hormones in experimental carcinogenesis, summarized the present view by stating that œstrogenic hormones and genetic factors have independent ætiological significance in several types of experimentally induced lymphoid, mammary, testicular, and hypophyseal tumours of mice.

In the period from 1935 to 1942, the hormones of the adrenals have been isolated by Reichstein, Kendall, and *Wintersteiner*, who demonstrated that the adrenal hormones were closely related in structure to the male and female sex hormones (review by Reichstein and Shoppee, 1943). During the same period, it was established that the adrenal hormones were necessary for life maintenance, and in addition were intimately concerned with protein, carbohydrate and electrolyte metabolism (Ingle, 1950). They influence the lymphatic tissues in a very significant way. Dougherty and White (1943) demonstrated involution of lymphatic tissues when adrenal function was increased and showed that this effect was coincident with nitrogen loss (White, 1950). In the same period, Kenyon (1944) and Kochakian (1950) demonstrated that the sex hormones functioned in the anabolic phase of nitrogen metabolism, while Albright (1947) showed that the female sex hormones play an important role in calcium metabolism and bone formation. These were notable discoveries since they demonstrated that the biological activity of the "sex steroids" was more far reaching than their influence on the secondary sex characteristics. The metabolic function of these hormones emphasizes a dual role in their action.

In 1944, Heilman and Kendall demonstrated that an adrenal hormone, cortisone, produced regression of lymphoid tumour tissue in mice, and Murphy and Sturm (1943) found a decreased susceptibility to transplanted leukæmias under the influence of adrenal hormones. Dalton (1944) found evidence of morphological changes in the adrenal gland with the development of tumours. These observations indicated that steroid hormones influenced abnormal growth. It may be suggested that the animal does not produce enough hormone to prevent tumour formation and that this failure of hormone production is due to a disturbed or decreased adrenal function in these animals. This problem has not been well studied with laboratory animals, but in our own investigation, which I will discuss later at this meeting, we were able to show that adrenal function is abnormal in many patients with neoplastic

growth. In 1944, not enough adrenal steroids were available to test the concept that adrenal hormones would be useful in humans with disease of the lymphatic tissues. The hope of obtaining large enough amounts of the adrenal stimulating hormone, ACTH, the isolation of which was reported at this time by Li *et al.* (1943) and Sayers *et al.* (1943) failed because industry was engaged in the emergency of war and because there was no promise of a cancer cure.

There remained, however, the possibility that hormonal imbalance might be involved in the production and growth of tumours. This hypothesis was supported by the work of Woolley (1950), who observed that endocrine imbalance produced by gonadectomy resulted in a nodular hyperplasia of the adrenal cortex that in certain instances led to adrenal tumours. The nodular hyperplasia was accompanied by pituitary tumours in these animals. It is of great significance that the administration of either male or female sex hormones to these gonadectomized mice *before* adrenal hyperplasia occurred prevented the adrenal and pituitary tumours. This close relation of endocrine imbalance, tumour formation, *and prevention by steroid hormone administration*, was supported and extended by the observation of Gardner (1947) that in certain low tumour strains of mice, testicular, pituitary, lymphoid, cervical and mammary tumours occur after œstrogen administration and that the incidence of these tumours can be decreased by the simultaneous administration of testosterone. Consequently, the major conclusion may be drawn that carcinogenesis is dependent on hormonal imbalance of several origins, involving a disturbed function of the gonads, adrenals, and pituitary glands.

The evaluation of endocrine factors in the ætiology of human cancer is much more difficult, since experimental conditions cannot be made equal to those available with animals. The hormones are differently metabolized in humans and in lower animals, and it may well be that their function and interplay is similarly variable. That the steroid hormones play a role in the ætiology of human cancer is strongly

suggested by certain observations, for example, the failure of eunuchoids and eunuchs to acquire prostate cancer is probably due to the lack of testicular hormone production (Moore, 1944). The incidence of human cancer increases at the time of endocrine changes, especially near the menopause in females. The age incidence in human cancer may well be linked with demonstrable endocrine imbalance or deficiency.

We therefore investigated in patients with neoplastic disease whether there were quantitative and qualitative changes in steroid excretion, in order to establish the facts of hormone production (see literature cited on p. 222). The results were compared with those obtained from normal subjects of the same age group. We concluded from our results that a decreased gonadal function and an abnormal adrenal secretion existed when cancer was present. This abnormal glandular function was established in three patients more than three years *before* cancer was diagnosed. If we look at these facts, it is clear that the decrease of gonadal and adrenal hormones is of major importance in cancer and that to influence the disease, it is necessary to restore a more normal glandular function.

As a consequence, substitution therapy was applied in the hope of restoring a normal hormone balance, and thus influencing abnormal growth. The administration of œstrogen and androgen in cancer of the breast (Nathanson, 1947) and the withdrawal of hormones by gonadectomy and adrenalectomy in cancer of the prostate (Huggins, 1946, 1950) have been reported as beneficial in these diseases. The mechanism of action on the abnormal process may be partly direct local action on the tumour as well as the profound general effect of these hormones on proteins and electrolyte metabolism in normal and abnormal tissues.

Another factor to be considered is the change these hormones cause in the endogenous production of the glands, which may in turn influence the neoplastic growth. Changes in endogenous production of hormones can be illustrated by three examples (Dobriner and Lieberman, 1950): (1) The prolonged

administration of testosterone changes the endogenous production of the precursors of androsterone and ætiocholanolone, since the urinary level of these metabolites decreased markedly after the treatment (Fig. 1). (2) The prolonged administra-

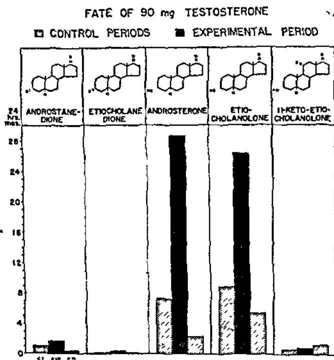


FIG. 1. The quantitative excretion of five steroid metabolites [illegible]

=post-testosterone control period.

tion of very small amounts of adrenal extract decreased the production of both 11-desoxy and 11-oxygenated hormones (Fig. 2). (3) Removal of the testes decreased the excretion of *androsterone* and *ætiocholanolone*, the metabolites of the

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apparent from the known reactions of these hormones on lymphoid tissues that the major attempts in humans should be directed toward disease of the lymphatic organs. The clinical aspects of this work were conducted by Dr. Pearson, Eliel, Burchenal and others* (Pearson *et al.*, 1949; Pearson and Eliel, 1950, 1951; Pillers *et al.*, 1951). The complete metabolic balance studies were made by Dr. Pearson, Eliel and others in the department of clinical investigation

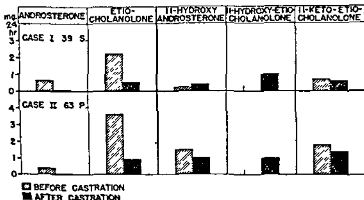


FIG. 3. The effect of orchiectomy on the excretion of five steroid metabolites by two men with cancer of the breast The values are expressed in milligrams per 24 hours.

(Pearson and Eliel, 1951), and in many of these patients the steroid excretion patterns were established, as I shall show later in this conference.

About 70 patients have been treated with 100 to 500 mg. of cortisone, or 100 to 400 mg. of ACTH per day for various lengths of time. Marked temporary regressions occurred in chronic lymphatic leukæmia, lymphosarcoma, acute lymphatic or granulocytic leukæmia, plasma cell myeloma and Hodgkin's disease. No effects were observed in patients with chronic myelogenous leukæmia, acute microcytic leukæmia, Ewing tumour, neuroblastoma, malignant melanoma and

*Dr. Burchenal reports the clinical findings on p. 198

male sex hormone as well as adrenal steroids (Fig. 3). These compensatory changes in hormone production resulting from the administration of steroids or from the withdrawal of one of the producing glands are probably mediated through

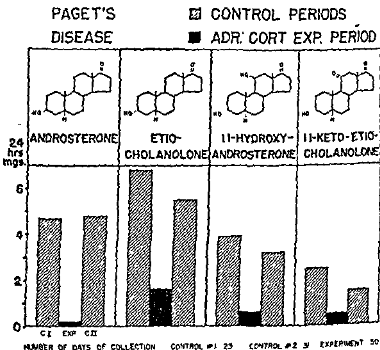


Fig. 3. The quantitative excretion of five steroid metabolites

changes in production of trophic hormones in the pituitary gland.

As the adrenal hormones as well as ACTH have been available since the fall of 1949, the opportunity was at hand to investigate their effect on neoplastic growth. It was

Tannenbaum (1947) in particular has emphasized the role of inanition in the growth rate of tumours. Tumour growth under high caloric intake can be viewed as a specific hormone starvation of neoplastic tissue, in that a large amount of the hormone participates in the elevated metabolism of tissues and is thus not available to the neoplasm. This is certainly suggested by the experiments of Pearson and Eliel (1950). If the hormonal supply were sufficient for the whole animal or could be applied *only* to the tumour tissue, it is easily possible to imagine the tumour shrinking to the vanishing point, even with excessive nitrogen intake. Conversely, if there had been enough hormones available at all times, the tumour might never have had the opportunity to come into existence.

From the clinical results and from the knowledge of hormone action, we can draw three significant conclusions: (1) Adrenal hormones, through their effects upon nitrogen metabolism, can be used to interfere with the progress of a tumour. If endogenous steroid production is adequate, or proper substitution therapy is undertaken, it is quite possible that certain tumours would not develop. (2) The change in hormone production and balance at present can only be detected in its subtle initial stages by study and examination of the urinary steroids. These substances therefore provide one of our few indices of physiological change associated with tumour incidence. (3) The "sex" hormones may be implicated in the cause of some tumours, especially neoplasia associated with reproductive organs. These same hormones unquestionably influence the course of the tumour's progression, and further study of their action in disease offers hope for a better therapy and possibly the prevention of abnormal growth.

I would like to express my deep appreciation to Dr Thomas F. Gallagher for his valuable suggestions and generous assistance in the preparation of this manuscript.

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several types of sarcoma and carcinoma. No differences have been observed in the clinical effects of administered cortisone or the hormones produced by the patient's own adrenals under the stimulation of ACTH, *provided that the adrenals responded adequately to the stimulus.* The therapeutic effects, although temporary, were thus restricted to the lymphatic tissues, and this was not an unexpected result in view of the biological action of the adrenal hormones. The necessity for large amounts of hormones was also not surprising, since it seems that the *normal adrenal produces* much larger amounts of hormones than were anticipated from purely a *priori* consideration.

There are three points in the metabolic studies of Pearson and Eliel that deserve special comment. *First:* They found that adrenal hormone administration in chronic lymphatic leukæmia with large tumour masses caused a disproportionate excretion of phosphorus in comparison with nitrogen. This finding is of great importance. It proved objectively that lymphatic tumour tissue was being destroyed, since lymphoid tissues are characterized by a high ratio of phosphorus to nitrogen. *Second:* After the combined administration of cortisone and testosterone in several patients with chronic lymphatic leukæmia with large tumour masses, the tumours shrunk considerably, and the patients remained in nitrogen balance. The phosphorus excretion continued at a high level, however, and the patients were in negative phosphorus balance. This observation indicates that the adrenal hormone under certain conditions *acts specifically* on tumour tissue, since the tumour regressed whereas the catabolism of other tissues was counteracted by the anabolic action of testosterone. *Third:* It would seem that the beneficial effect of cortisone is dependent on dietary intake. One patient, who showed a decrease of tumour masses during the administration of cortisone and exhibited a markedly negative nitrogen and phosphorus balance, was given twice the amount of food. The nitrogen and phosphorus balance then became slightly positive, and no further shrinkage of the tumour occurred.

DOBRINER: There was a slight increase in phosphorus excretion but t was not significant. It is a difficult thing to understand because tissue s being broken down and yet the products are not excreted. They nust be used for rebuilding.

BOYLAND: It would be interesting to know the nature of the excess nitrogen excretion. One might expect it to be derived from nucleic acids, and that there would be an excess of uric acid.

DOBRINER: There is in some patients and not in others.

KELLIE: It seems to me that the importance that was formerly laid

DOBRINER This is somewhat outside of my personal field

BEGG: Nucleoproteins are synthesized but uric acid comes from a breakdown of the nucleoprotein residues.

BEGG: Yes, but via the purine system We can undoubtedly say that

lished.

DOBRINER: It will take a long time because this is very tedious and time-consuming work.

KELLIE: Is there any definite information whether Compound F has effects which are not present with Compound E, or vice versa? Or can one assume that Compound E will be reduced to Compound F?

DOBRINER: I will show this to you later. It is a very simple metabolic change and it seems to occur all the time. The substance produced by the adrenals under ACTH stimulation is certainly not Compound E; it is Compound F

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DISCUSSION

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excretion there:

with a million cells intraperitoneally the control mice develop tremendous spleens and large lymph nodes. The spleen from one of these untreated animals weighed 1,550 mg. The spleen from a mouse which had been treated for 17 days with cortisone at 50 mg. per kg. three times daily (which is a toxic dose) weighed only about 65 mg. The nodes could not be found, and sections of the spleen and liver showed no leukæmic infiltration. This strain of leukæmia in the C_{58} stock of mice (which is not our standard strain) has been of use, and we hope to be able to get a dose which will prolong the survival time of these animals.

On the other hand, acute leukæmia in man is a different story. Dr. Dobriner has given you an account of the reasons that led up to the trial of ACTH in acute leukæmia by Dr. Pearson and, I believe independently at about the same time, by Dr. Farber of Boston. Today I am speaking here, partially for myself, but mainly for Dr. Pearson. Dr. Pearson has treated some 31 cases of acute leukæmia and did the original work and the metabolic work on the study of this disease. Our group has been interested in it more from the therapeutic trials aspect, and we have treated an additional 21 cases with cortisone, so that altogether we have treated some 51 cases of acute leukæmia. In Dr. Pearson's group 20 children were treated with these two compounds, and nine of those had good remissions.

Of 10 adults, four responded well. In our series of 15 children, nine did well, and of six adults, only one responded well. In other words, out of 35 cases in children, there were 18 good responses; out of 16 adults, five good responses.

I will discuss mainly our own results now. We got real remissions, hæmatological remissions with the peripheral blood returning approximately to normal, liver and spleen going down to normal size, nodes going down, and with a marrow which returned approximately to normal. I do not say that at the best of the remissions one couldn't find a leukæmic cell. I'm sure that, if a hæmatologist looked at it and knew it was a case of leukæmia, he could find leukæmic cells in that

ACTH AND CORTISONE IN ACUTE LEUKÆMIA IN CHILDREN

J. H. BURCHENAL

I AM going to talk mainly about the therapeutic effects of cortisone and ACTH on acute leukæmia.

I would like to say a word about the advantages of using acute leukæmia for an assay method, a method of testing compounds. Dr. Huggins mentioned this morning that the clinician always has difficulty in assessing the value of a compound in the patient unless it is a truly curative compound, which obviously none of these are. Biopsies can be done occasionally, but regular biopsies are hard to get unless you have a very co-operative patient. In acute leukæmia, by sticking the patient's finger or by perhaps doing a sternal puncture, you can get a biopsy of the tumour at any time that you wish. Also you have a disease which runs a rather acute, fulminating course, which has some spontaneous remissions, but not very many, and which rarely shows two spontaneous remissions in an untreated patient. Moreover, it occurs fairly commonly in children, and children appear to be able to stand the effects of substances such as ACTH and cortisone better than older people, particularly the aged.

There have been difficulties in using mouse leukæmia as a screening procedure for the steroids because the leukæmia which we have been using has been very acute, and has not been affected by ordinary doses of cortisone; with the maximum tolerated dose of cortisone or ACTH we cannot prolong the life of these animals. However, these compounds do have some effect on the leukæmia, because if we give large doses, supralethal doses, the animals will die at the same time as the control animals, but they will not die of leukæmia; they will have no infiltration. Nineteen days after inoculation

Fig 1. *Marrow of patient* with acute leukæmia, after treatment with 50 mg cortisone per day for 28 days

Fig. 2 Same patient as in Fig. 1 After treatment with 400 mg

marrow, but they have decreased to a very small percentage. On the other hand, I don't believe that a hæmatologist seeing those marrows, without knowing that the patient had leukæmia, could make the diagnosis. In a few cases, however, we have had hæmatological remissions in which the marrow improved markedly, but the patient did *not*. I've not seen that with other drugs in acute leukæmia.

Figs. 1 and 2 show an example of what we mean by a good hæmatological remission. Fig. 1 shows the marrow of a patient with acute leukæmia after he had been treated with a little cortisone. His marrow was approximately that way before any treatment at all. He had 50 mg. a day for 28 days, an insufficient amount so far as he was concerned. As you can see, his marrow was made up all of one type of cell, stem cells of acute leukæmia.

After he had been treated with 400 mg. of cortisone a day the cell picture (Fig. 2), instead of being uniform as it was before, showed nucleated red cells, myelocytes, metamyelocytes, and polymorphonuclears, the more mature cells of the myeloid series.

I would like to discuss a case that is typical of those we have been treating. This is of a seven-year old girl who had been treated with other drugs for some time before and had responded to them, but had eventually failed to respond to anything. Her bone marrow showed 87 per cent stem cells. Her white count was rather low, down around 5,000, but the percentage of abnormal cells was almost 100 per cent. She was then treated with cortisone. There was a slight rise at first in the white count and then a fall, and the thing that is really important is that the percentage of stem cells decreased to 10 per cent, and that the marrow showed essentially a normal picture. Stem cells, of course, should not be present in a normal marrow in any appreciable quantity, but it's very difficult to differentiate between lymphoid cells and stem cells, particularly in children, so that it is possible that that figure may be 16 per cent lymphocytes, which is not too far from normal. During the time that she was improving the

eosinophils fell, and the reticulocytes rose slightly to about 5 per cent. She had one transfusion which built up her hæmoglobin and after that she maintained it quite well. However, after stopping treatment for a short while, there was a rapid reversion to an abnormal marrow picture. She was started on treatment with antifolics, to which she was resistant and had never responded, and there was no more response. The marrow became worse. She was brought in, transfused, because at that stage she was in rather poor shape, and then she was treated again with cortisone. The marrow again responded, going from 90 per cent abnormal cells down to 11 per cent, with a fall in the eosinophils and with a marked rise in reticulocytes, up to about 15 per cent. After her second course, she was in quite good shape, and had not shown too many undesirable side effects. She put on some weight, but she did not show particularly spindly legs, nor a very large abdomen, and her face, although fuller, was not as full as it might have been.

We have seen remissions lasting anywhere from one to twelve weeks. In many of these cases the marrow relapses although the patient still feels well, perhaps for as long as a month after. We usually start treatment, if possible, as soon as a real relapse in the marrow shows up.

We have usually given ACTH four times daily in doses of 50–100 mg. a day for children and 100–200 mg. a day for adults, but we have gone up to 400 mg. a day in some patients, and above that in one or two. ACTH is given four times daily because it has been shown that ACTH goes in and out of the adrenals fairly rapidly.

In Dr. Pearson's group cortisone has been given the same way, every six hours. The dosage has been a little higher, running from 50 mg. to 100 or 200 mg. in children, and in adults, from 200 to 400 mg. in a total daily dose. Because we were *interested in treating* some patients as outpatients because of lack of beds, we tried giving these doses in a single daily injection and I think that our results have been reason-

occurred particularly in children. It comes on sometimes rapidly, sometimes after a considerable dosage, and we have found no way of getting away from it. It seems that if the blood pressure goes up to high levels or if episodes of hypertensive encephalopathy with loss of consciousness intervene, it is best to stop the drug.

Psychoses have been seen in a certain number of patients and we feel that if there is any real mental aberration, it is best to stop the drug. Some of these patients have committed suicide. Others have died during treatment, but presumably of their disease rather than of the psychosis. Others are still being treated for the psychoses with no sign of improvement. We have a psychiatrist who watches these patients fairly closely, and when he feels there is any abnormality appearing, we generally stop treatment. Sometimes, however, the psychoses come on very rapidly. Patients who are watched very closely may seem a little agitated one morning and suddenly commit suicide the next day. We have not been impressed by psychiatric changes in children as yet. It's a little hard to assess that because the children come from all sorts of environments. Their personality changes a certain amount while they're in a ward, even without treatment, because they are away from their normal environment and they get bouts of homesickness. We have not yet seen any frank psychoses in children.

We have been very impressed by the occasional incidence of overwhelming infections in these patients, mostly patients who have had prolonged treatment with one of the steroids. Of course, in leukæmics one normally expects to get infections, but not in a leukæmic whose bone marrow has gone back to normal, as in some of these patients who had good hæmatological remissions, but no clinical remissions. Dr. Pearson's and our groups both feel that there is very definitely, in some of these cases, an increased susceptibility to infection. If there is an infection and if the antibiotics which are used in large doses don't seem to be able to handle it rapidly, then we feel it is best to stop the steroids.

make sure that a patient who responds well to 200 mg. in a single dose would not respond to perhaps 100 mg. if the dose were divided, and I think it's very possible that divided dosage may be better. I can say, however, that in a child given a single daily dose of 150 or 200 mg., definite beneficial effects can be obtained. In some of the patients who have died under treatment, we have seen large depots of cortisone in the muscles. Since cortisone is relatively insoluble, probably one gets a depot effect, and a single injection a day may be sufficient.

Dr. Dobriner has gone over the undesirable side effects that occur with these drugs. Sodium retention with water retention and œdema is certainly important. From a therapeutic point of view that can be handled quite well by putting the patient on an almost salt-free diet, or at least a low sodium diet.

Weight gain, aside from œdema, occurs if these patients are allowed to eat as much as they want. We try to calculate what the child should eat if he were in normal health, and put him on a diet limited to that number of calories. Before he's getting cortisone or ACTH he won't eat that much, but after he gets these drugs he rapidly develops a ravenous appetite and reaches the ceiling imposed by his diet. Then if you can watch the other children and make sure he doesn't steal food from them, on that limited food intake and low sodium diet there is usually no weight gain.

Metabolic alkalosis has to be watched for, but when it develops it can be corrected by the administration of potassium chloride.

In patients who have been treated for a long period of time with ACTH or cortisone we have seen development of Cushing's facies, a certain amount of hirsutism, and acne. These symptoms, although bothersome to the patient and occasionally to the family, have not been a contra-indication to further therapy.

There are, *however, three difficulties that* have not been surmounted as yet. The first is a rise in blood pressure, with or without hypertensive encephalopathy. That has

for about four weeks and then he came in and the marrow was all bad again. He was started on the same dose of ACTH that he had before, and there wasn't any change in the marrow at all. He was then given larger doses of ACTH, up to 400 mg. per day and I think he was also given cortisone just before death, but there was no improvement the second time, no change in the bone marrow, no improvement in nucleated red cells. The differential, which went bad a little more slowly than the marrow differential, still stayed completely bad, and the white count, which had started going up in his last relapse, continued to go right on up and was around 870,000 at the time of death. So that there was absolutely no clinical response, although metabolically he was showing some response, as shown by his increased excretion of steroids.

Now we come to the practical value of these compounds. We are looking for a compound that will work when the folic acid antagonists do not work, because we have a large number of patients under treatment constantly, and we know that sooner or later the folic acid antagonists are going to cease to work. An example of this is L.D., a five-year old boy who had been treated since December 1948 with folic acid antagonist and had repeated responses, but finally reached a stage in March 1950 where he was totally resistant to the antifolics. He was then treated with 150 mg. of cortisone a day. He had an episode of hypertensive encephalopathy which alarmed us; at first we thought he had had a cerebral accident, but he had not, and we stopped treatment for a short while and then started again. His blood pressure did not go up too markedly, and we felt that it was do or die with him so we carried on the therapy. Eventually he got a response. His stem cells decreased down to about 30 per cent. That is not a perfect marrow by any means, but the percentage of erythroid elements increased to 55 per cent of all the marrow cells, and he built hæmoglobin. He had a small transfusion, but the rise in hæmoglobin was not due to that; it was due to his building new red cells of his own. His reticulocytes

As to the repeatability of these remissions in children, we have had six who have had a second course of therapy and who have responded. Two of these, from one of whom I showed you a marrow slide, had just as good remissions with the same amount of the drug as they did the first time. On the other hand, four others required considerably more cortisone or ACTH the second time. We have pushed some of them into partial third remissions, but generally there is a falling off in the relative completeness of the remission and the ease with which it can be attained. They seem to develop a refractory state to this drug faster than a similar type of patient would do to the folic acid antagonists.

In adults, on the other hand, we have had two patients with excellent results the first time, who were treated a second time and neither showed any beneficial effects. I would like to discuss one of them, case RR, in more detail. He showed a beautiful response the first time; the second time, although he had had a metabolic response, his leukæmia was not affected. On his first course of therapy he started out with a white count of 100,000 cells, and after four or five days' treatment with ACTH his count was down to 2,000. This is one of the interesting things about ACTH. Ordinarily in treating chronic leukæmia with the nitrogen mustards or acute leukæmia with the antifolics, if we get a drop from 100,000 down to 2,000, we would be alarmed and would stop therapy, but this therapy was carried right on and the count levelled off at 2,000, didn't go down to 200 or 20 cells, and the patient got a tremendous and very rapid clinical betterment. He was a young doctor who knew the situation and perhaps there was some psychical aspect, though it was hard to see any. He had considerable bone pain when he came in. Within 12 hours of getting his first injection of ACTH that had eased. There was rapid improvement in the myeloid cells of the peripheral blood and in the marrow. The erythroid activity increased from practically nothing up to 60 nucleated red cells per 100 white cells.

After the drug was stopped the marrow remained normal

some transient decrease in the nodes or in the masses, which is very real but which is not as prolonged as with X-ray therapy. In the cases where the patient is X-ray resistant and moribund, then this agent is no better than HN2.

We also tried Compound A, 125 mg. given four times per day. It was administered for 22 days in a case of acute leukæmia. The patient showed an increased weight and œdema, but there were no beneficial effects.

We are now trying Compound L. It has been administered in dosages of 150 mg., twice daily, but we have no results as yet.

DISCUSSION

GARDNER: The laboratory tests have been on transplanted leukæmia

very large numbers of mice.

GARDNER: *Do you think that there might be a certain point of similarity between the resistance to the second and third treatment in the human cases and the prolonged survival of the cells in the animals that had transplanted leukæmia?*

BURCHENAL: I don't know. But we do have experiments underway with the Patterson lymphosarcoma and with the Wagner osteogenic

BEGG: In what per cent of cases did you give transfusions and antibiotics?

BURCHENAL: We tried to stay away from transfusions as much as possible, although on the other hand, if the patient looked in bad shape, we didn't hesitate to give them. We've got a base line of 150 cases that were treated with transfusions and a certain number of antibiotics, but there always is a possibility that if you put a large amount of blood in there that you may get some temporary remission. We have had to use antibiotics fairly frequently because of infection.

BEGG: You have some cases there in which you have response without transfusion?

went up markedly. When I left we had just started him off on antifolic therapy, hoping that he might respond again to it.

We have also tried the converse, using the antifolics on patients who have become resistant to cortisone or steroid therapy. Three of those five or six patients have responded well to the antifolics.

I will try to compare briefly the values of these compounds with some of the known therapeutic agents in cancer. X-ray, of course, is useless in acute leukæmia, and HN2 and the various mustards are of no real benefit. The antifolics are useful in some cases; in children 30–40 per cent remissions can be achieved. With the antifolics there is very little beneficial effect in adults, and it takes longer to get results. It would appear that the steroids and ACTH work faster in the acute leukæmias, and I believe that you probably have more chance of helping a very sick patient with ACTH or cortisone than you have with the antifolics. Once you get him out of his sick situation, and if you do get a remission with either compound, I think you have more chance of getting many remissions with the antifolics than you do with the steroids. With the latter the remissions are not likely to persist as long or to be as repeatable.

There is a marked contrast between chronic lymphocytic leukæmia and the acute leukæmias As Dr. Dobriner has told you, if you treat a case of chronic lymphocytic leukæmia with cortisone or ACTH, the spleen decreases in size, the nodes decrease, the liver decreases, but the white count goes up tremendously, from 100,000 or so to as high as 800,000. We've seen one case who went from 700,000 to 1,200,000. On stopping the dosage, surprisingly enough, the count falls. On the other hand, this drug does seem to have some beneficial effect in patients who, if treated with X-ray, might well develop thrombocytopenia, anæmia, and so on.

There seems to be some suggestion of stimulation of the erythroid elements, so it has usefulness there compared with HN2. In Hodgkin's disease and in lymphosarcoma there is

some transient decrease in the nodes or in the masses, which is very real but which is not as prolonged as with X-ray therapy. In the cases where the patient is X-ray resistant and moribund, then this agent is no better than HN2.

We also tried Compound A, 125 mg. given four times per day. It was administered for 22 days in a case of acute leukæmia. The patient showed an increased weight and œdema, but there were no beneficial effects.

We are now trying Compound L. It has been administered in dosages of 150 mg., twice daily, but we have no results as yet.

DISCUSSION

GARDNER. The laboratory tests have been on transplanted leukæmia rather than on the spontaneous disease in all cases so far, have they not?

[illegible]

very large numbers of mice.

GARDNER: Do you think that there might be a certain point of similarity between the resistance to the second and third treatment in the human cases and the prolonged survival of the cells in the animals that had transplanted leukæmia?

BURCHENAL: I don't know. But we do have experiments underway with the Patterson lymphosarcoma and with the Wagner osteogenic

[illegible]

BEGG: In what per cent of cases did you give transfusions and antibiotics?

BURCHENAL: We tried to stay away from transfusions as much as possible, although on the other hand, if the patient looked in bad shape, we didn't hesitate to give them. We've got a base line of 150 cases that were treated with transfusions and a certain number of antibiotics, but there always is a possibility that if you put a large

[illegible]

Burchenal: Some cases in our series have had no transfusion and

that it was neoplastic tissue, or at least lymphoid tissue, that was being destroyed. Wouldn't you think that was so, Dr. Dobriner?

Dobriner: Yes. I think it is good metabolic evidence.

Burchenal: In chronic lymphocytic leukæmia we find that we can continue to get remissions on repeated treatment. After Dr. Pearson treats his chronic lymphocytic leukæmias and gets a response, they do well for about three months and then the nodes start coming up again. He has treated them again and again. One of them has been treated for 15 months at least, and the responses are approximately as good now as they were to start out with. These were all adults. It seems rather surprising to me that the chronic lymphocytic leukæmias should continue to respond just as well, whereas the acute leukæmias in adults do

However, we are trying now to wait a little longer instead of giving

Stock: No.

in these cells.

definitely stimulated, as shown by the reticulocytes.

Begg: What about the effect of antibodies to ACTH? Is there

effect. It looks as though the resistance there were right in the leukæmic cell. There have been reports in the literature of animals getting resistant to it, but as far as I know this has not been demonstrated in man.

BOYLAND: Have you tried simultaneous treatment with antifolic compounds and cortisone?

BURCHENAL: We have not tried that to the full, because we felt that if we did get a response we wouldn't know which it was due to. We tried it out and it hasn't seemed to be any better than either one. Dr. Farber does treat a lot of his cases that way. He treats them with various regimes. One time he treats for perhaps 5 or 6 days with ACTH, and then puts them over on the antifolic and another time he will treat with the two of them simultaneously.

SOMMERVILLE: I was surprised to hear that you got hypertension with both cortisone and ACTH. In cases where you gave ACTH for prolonged periods, did you find that the electrolyte change that was remarked earlier in potassium output and chloride retention was maintained?

BURCHENAL: We usually put most of the patients on potassium chloride.

SOMMERVILLE: Does ACTH cause hypertension in normal people and cortisone not?

DOBRINER: No.

BOMFORD: What effect do these substances have on the bone marrow of patients without this particular condition?

BURCHENAL: I can't tell you. They have used it in pernicious anæmia without leukæmia, and I believe there is some improvement in the erythropoietic elements in cases of Hodgkin's disease, where there is no real damage to the bone marrow.

BEGG: Hench and his group reported in the *Archives of Medicine* in February, that they definitely got improvement in hæmoglobin in their rheumatoid cases.

BOMFORD: There was no depression of white cell formation?

BURCHENAL: I don't think so. You very seldom get an aplastic marrow as you do with the mustards or with X-rays.

LYONS: Did you get any inflammatory reactions at the sites of the injections, especially after the second or third injection of ACTH?

BURCHENAL: It depends on the patient. We have seen patients who could tolerate large doses of it for a long period of time without any trouble at all, mainly children with very little musculature.

LYONS: Do you try sensitivity reactions before you start the patient?

BURCHENAL: No, we haven't done that.

LYONS: I wondered if it could be connected with the development of antibodies, as Dr Begg suggested.

BURCHENAL: I think it is more likely to be a question of poor absorption in some of these patients.

LYONS: Do you recover any of this ACTH in the urine?

DOBRINER: We have never tried

ADRENAL FUNCTION AND STEROID EXCRETION IN NEOPLASTIC DISEASE

KONRAD DOBRINER

THIS morning I have discussed the correlation of steroid hormones with cancer. I pointed out that the study of steroid hormone production in normal and diseased states is possible by a detailed investigation of steroid excretion in urine, if one makes the reasonable assumption that steroid excretion is a measure of the hormone production of the adrenals and gonads. Since 1940 our investigations have been directed toward the development of methods for the study of steroid excretion patterns, with the aim of understanding the relationship between endocrine function and diseased states, with special emphasis on neoplastic disease (Dobriner, 1948, Reifenstein, Homburger, and Dobriner, 1950; Dobriner and Lieberman, 1950). We have thus far developed our procedure to its present use which requires a one day urine sample. By means of careful chromatography after chemical separation of the principal classes of steroids, we are able to obtain all or very nearly all the ketosteroids as single pure compounds or binary mixtures (Dobriner, Lieberman and Rhoads, 1948; Lieberman *et al.*, 1948; Lieberman, Fukushima and Dobriner, 1950). By the systematic application of infrared spectrometry on a micro scale, we can define the pure compounds or simple mixtures (Dobriner, Lieberman, Rhoads, Jones and Williams, 1948; Jones and Dobriner, 1949). Quantitative analysis by microcolorimetry then permits the determination of the amount of each steroid excreted during the twenty-four interval.

If we examine the steroid excretion patterns of normal males and females (Fig. 1) we find that there are four compounds that make up the major part of the ketosteroids.

Androsterone and its isomer ætiocholanolone are two meta bolites of gonadal and adrenal hormones of the C-11 deox type like testosterone and compound S (Reichstein), wherea 11-hydroxyandrosterone and 11-ketoætiocholanolone ar derived from adrenal cortical hormones with a C-11 oxyge

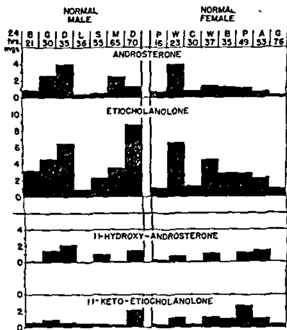


Fig. 1. Steroid excretion patterns of four compounds in normal male and female subjects.

function, like compound F and cortisone. In addition to thes major metabolites, small amounts of many other steroid are excreted, including compounds F and E. In norma subjects there are no qualitative deviations from the norma pattern, but the amount of each compound varies somewha in different individuals, just as there are *quantitative* difference in any metabolic process. One may say that the steroid

patterns indicate the *individual* hormone production and that this varies between individuals just as there are differences in height, weight, or temperament.

The steroid pattern is quite abnormal in patients with neoplastic disease, as seen in Fig. 2, where a comparison has been made with the excretion levels of normal subjects in

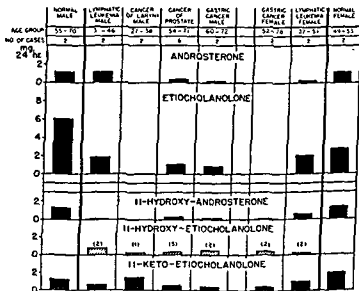


FIG. 2. Comparison of steroid excretion patterns in patients with neoplastic disease, and in normal subjects of the same age group

similar age groups. In contrast to the pattern shown for the normals (Fig. 1), there are both *quantitative* and *qualitative* differences in steroid excretion, whereas in the normals *only quantitative* changes are found. The decrease in the steroid excretion of cancer patients is especially marked with 11-deoxy type of steroids; for example, androsterone and ætiocholanolone were not excreted by male patients with cancer of the larynx nor by female patients with gastric cancer. In the other types of neoplastic growth, the excretion of these two

compounds was significantly decreased. A similar but somewhat smaller decrease in the excretion of 11-oxygenated compounds (11-hydroxyandrosterone and 11-ketoætiocholanolone) was observed. The *qualitative difference* is of much greater significance, since in the patients with neoplastic disease *a new compound is excreted*, 11-hydroxy-

COMPOUND F	COMPOUND S	TESTOSTERONE	
			HORMONES
11-OH-ETIOCHOLANOLONE	17 OH-PREGNANOLONE	ETIOCHOLANOLONE	METABOLITES
11-OH-ANDROSTERONE		ANDROSTERONE	

FIG. 3 Metabolic relations of hormones and their urinary metabolites.

ætiocholanolone, which from its chemical structure appears to be a metabolite of adrenal cortical hormones with a C-11 oxygen function. The metabolic relations of precursor hormone and excreted steroids are shown in Fig 3. 11-Hydroxyætiocholanolone was found in only two instances in a survey of 28 normal males and females. The two "normal" persons are over 70 years of age and have as yet no detectable evidence of neoplastic or other disease. Since the compound

occurs in the urine of some patients with essential hypertension and rheumatoid arthritis, its presence is not specific for cancer. 11-Hydroxyætiocholanolone is consistently excreted by patients with Cushing's syndrome, a disorder due to adrenal cortical hyperfunction with increased production of adrenal hormones of compound F and E type. The compound was also observed in the urine after stimulation of the adrenals by ACTH and after administration of large amounts of cortisone. However, 11-hydroxyætiocholanolone was not found in the urine of patients with the adreno-genital syndrome, another disorder characterized by the production of large amounts of adrenal hormones. The metabolic and clinical significance of the excretion of 11-hydroxyætiocholanolone is that in patients with neoplastic disease there is an abnormal adrenal function, somewhat reminiscent of the Cushing syndrome in the type of steroid produced. Whether this is only a derangement in hormone production, or whether there is in addition a faulty metabolism of a normal hormone, must be decided by further investigations with labelled hormones. We believe, however, that our results definitely indicate a very pertinent role of the adrenal gland in cancer, as I discussed in some detail this morning. In addition to the abnormal adrenal function, there is a very marked decrease in gonadal hormone production, to such an extent that in certain instances no trace of the metabolites of hormones like testosterone can be found in urine. This decrease or lack of male sex hormone production suggests that in certain cancer patients we are dealing with a condition very similar to physiological castration.

Two problems then arise: are these changes in adrenal and gonadal function connected with the cause of the disease, or is the abnormal hormone production and metabolism a consequ[illegible] of the disease? Both questions are difficult to [illegible] ht that debilitation, so [illegible] have an influence on [illegible] laryn[illegible] is *not* the principal other typ[illegible] is well illustrated

in Fig. 4, where excretion patterns of patients with prostatic cancer are shown. This group of patients with cancer of the prostate was made up of men with and without debilitation. In all patients, independent of debilitation, the two major

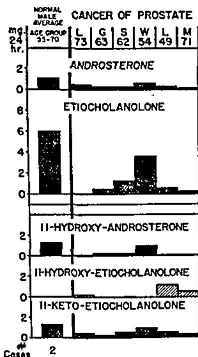


FIG. 4. Steroid excretion patterns in patients with cancer of the prostate and normal subjects of the same age group.

urinary metabolites androsterone and ætiocholanolone were markedly decreased in amount. The abnormal metabolite, 11-hydroxy-ætiocholanolone was present in all except one of these patients. I could give you many other illustrations to support the statement that general debilitation is not the cause of these changes in steroid excretion in cancer patients.

occurs in the urine of some patients with essential hypertension and rheumatoid arthritis, its presence is not specific for cancer. 11-Hydroxyætiocholanolone is consistently excreted by patients with Cushing's syndrome, a disorder due to adrenal cortical hyperfunction with increased production of adrenal hormones of compound F and E type. The compound was also observed in the urine after stimulation of the adrenals by ACTH and after administration of large amounts of cortisone. However, 11-hydroxyætiocholanolone was *not* found in the urine of patients with the adreno-genital syndrome, another disorder characterized by the production of large amounts of adrenal hormones. The metabolic and clinical significance of the excretion of 11-hydroxyætiocholanolone is that in patients with neoplastic disease there is an abnormal adrenal function, somewhat reminiscent of the Cushing syndrome in the type of steroid produced. Whether this is only a derangement in hormone production, or whether there is in addition a faulty metabolism of a normal hormone, must be decided by further investigations with labelled hormones. We believe, however, that our results definitely indicate a very pertinent role of the adrenal gland in cancer, as I discussed in some detail this morning. In addition to the abnormal adrenal function, there is a very marked decrease in gonadal hormone production, to such an extent that in certain instances no trace of the metabolites of hormones like testosterone can be found in urine. This decrease or lack of male sex hormone production suggests that in certain cancer patients we are dealing with a condition very similar to physiological castration.

Two problems then arise: are these changes in adrenal and gonadal function connected with the cause of the disease, or is the abnormal hormone production and metabolism a consequence of the disease? Both questions are difficult to answer. There seems to be no doubt that debilitation, so often a consequence of cancer, can have an influence on hormone production. That debilitation is *not* the principal reason for the change in steroid excretion is well illustrated

in her urine (period I). We have followed the steroid excretion pattern now for more than six years (period XI) after the recognition of her tumour, and in each period the abnormal steroid was present. It is now eight years after her mastectomy and no recurrence has been observed. This patient has not lost weight since the first urine collection, and, with the exception of increasing age, enjoys perfect health. This is a very good instance where debilitation can be excluded as the reason for an altered steroid excretion. I would like to mention another patient with aplastic anæmia of many years duration. She excreted the abnormal steroid 11-hydroxyætiocholanolone in a collection period four years before her death at a time when no cancer was diagnosed. The autopsy revealed, however, the existence of a cancer of the lung. These two instances certainly prove that the adrenal abnormality was present when cancer was not diagnosed. This adrenal disturbance seems to be present where early cancer may not be detected clinically, and may possibly exist in cases where cancer will develop at a later date. If this latter point could be proven, then *the cause of cancer would involve* a steroid hormonal factor and deranged adrenal function.

I would like to discuss another aspect of adrenal function in neoplastic disease. Can we draw any conclusions about the function of the adrenal glands when these are stimulated by ACTH? (Dobriner *et al.*, 1950, 1951*a*, 1951*b*; Dobriner, 1951). In six normal males and nine patients with neoplastic disease, the ketosteroids and formaldehydogenic steroid levels were measured during a control period and during a period of 12 days when each of the subjects received 100 mg. of ACTH daily in four divided doses. In five normal subjects the increase of ketosteroids and formaldehydogenic steroids was remarkably similar (Table I). In one subject, no increase in ketosteroids was observed, but there was an increase of formaldehydogenic steroid to a level within the range of other normal males. Similar lack of response to ACTH as far as the ketosteroid excretion is concerned has been observed by us in several patients with neoplastic disease. It is clear then

I should like to show you some evidence concerning the causal relation of cancer and hormone production (Fig. 5). We collected the urine of a middle-aged woman during several periods in the course of more than three years (period I, II,

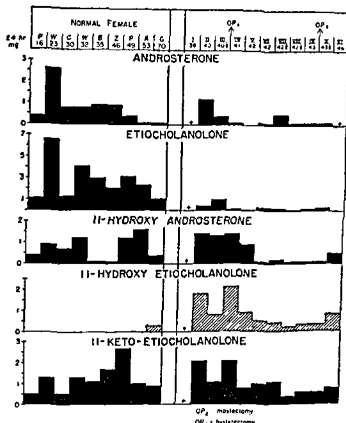


FIG. 5 Steroid excretion patterns of a patient with cancer of the breast before and after mastectomy.

and III) for the purpose of establishing steroid excretion patterns in her age group. Between period III and IV, cancer of the breast (grade 3) was diagnosed and a total mastectomy was performed. It can be seen that more than three years *before cancer was diagnosed* the abnormal steroid was present

metabolism and the status of adrenal function in neoplastic disease. In Fig. 6 the steroid excretion patterns of the most abundant urinary steroids before, during and after ACTH

Table II

EXCRETION OF KETOSTEROIDS AND FORMALDEHYDOGENIC STEROIDS BEFORE AND DURING ACTH INJECTIONS IN PATIENTS WITH NEOPLASTIC DISEASE

KS (mg /24 hr)		*FS (mg /24 hr)*	
Control	*12 Days ACTH*	*Control*	*12 Days ACTH*
14 7	63 6	4 2	11 9
16 6	42 7	1 7	37 8
17 0	41 7	5 4	24·0
24 7	32 6	1 0	18 3
16 5	38 6	2 4	25·5
10·7	48 6	—	—
16 0	60 8	10 5	40 4
15 1	55·0	0 2	11 9
17 1	74 9	5 0	43 0
Average			
16 5	50 9	3 8	26 6
Ratio			
1 0	3 1	1 0	7·0

Table III

COMPARISON OF EXCRETION OF KETOSTEROIDS AND FORMALDEHYDOGENIC STEROIDS BEFORE AND DURING ACTH INJECTIONS IN NORMAL SUBJECTS AND IN SUBJECTS WITH NEOPLASTIC DISEASE

	KS (mg /24 hr)		*FS (mg /24 hr)*	
	Control	*ACTH ST*	*Control*	*ACTH ST.*
	Average			
Normal (6)	*19 9*	*49 7*	*0 3*	*9 9*
Neoplastic Disease (9)	16 5	50 9	3 8	26 6
	Ratio			
Normal (6)	1 0	2·5	1 0	34 7
Neoplastic Disease (9)	1 0	3 1	1 0	7 0

that there is sometimes a changed or abnormal adrenal responsiveness to ACTH. If one compares the steroid excretion of the six normal subjects after ACTH with that of nine patients with neoplastic disease, one observes that the ketosteroid response of both groups is of the same order of

Table I

EXCRETION OF KETOSTEROIDS AND FORMALDEHYDOGENIC STEROIDS BEFORE AND DURING ACTH INJECTIONS IN NORMAL SUBJECTS

KS. (mg /24 hr)		FS (mg /24 hr)	
Control	*12 days ACTH*	*Control*	*12 days ACTH*
22 7	58 1	0·3	8 1
20·2	60·7	0 4	12·4
23 1	29 8	0·2	7·1
15·0	54 0	0 3	14 1
17 4	55 9	0 2	11·9
21 0	39·7	0 1	5·5
Average			
19 9	49 7	0·3	9·9
Ratio			
1·0	2 5	1·0	34·7

magnitude while, in contrast, there is a striking difference in the excretion of the formaldehydogenic steroids (Table II and III). During the control period in neoplastic patients, the formaldehydogenic steroid levels are higher than those in normal subjects. These findings indicate that the adrenals are already functioning at an increased level in the patients with neoplastic disease, and that their adrenals not only respond to ACTH but do so to a greater extent than the adrenals of normal subjects. This again indicates a different functional status of the adrenals in patients with neoplastic disease.

A study of the individual steroids excreted before and during stimulation of adrenal function by ACTH should give more detailed information about hormone production and

administration in patients with neoplasia is shown in comparison with the patterns of normal untreated subjects of the same age group. There was an increased excretion of both 11-oxygenated and 11-deoxysteroids during adrenal stimulation. It is important to note that this proves that the human adrenal produces at least two types of steroid hormones, one of the 11-oxygenated type like compound F (Kendall) and one of the 11-deoxy type, probably like compound S (Reichstein). On ACTH treatment the patients responded with different amounts of some steroids, and in addition new compounds appeared. These were not the same in all subjects. The results indicate qualitative and quantitative differences in the responsiveness of individual patients to adrenal stimulation.

We conclude from the evidence presented that both gonadal and adrenal functions are impaired in patients with neoplastic disease. There is evidence suggesting that the adrenal glands of these patients with neoplastic disease function differently both *qualitatively* and *quantitatively* from normal glands. There is a decrease in excretion of the metabolites of C-11 deoxy hormones, and some metabolites of the C-11 oxygenated steroids are qualitatively different from those of normal subjects. The excretion of C-11 oxygenated adrenal hormone metabolites is at a fairly normal or slightly increased level, but not at the maximum level to which the gland can be stimulated. One may speculate that not only an abnormal gland but also a changed pituitary adrenal regulation is present in neoplastic disease. The adrenal hormones play an important role in the course of the disease and may be involved in its cause.

I would like to express my deep appreciation to Dr. Thomas F. Gallagher for his generous and valuable assistance in the preparation of this manuscript.

This investigation was aided by grants from the American Cancer Society (on recommendation of the Committee on Growth of the

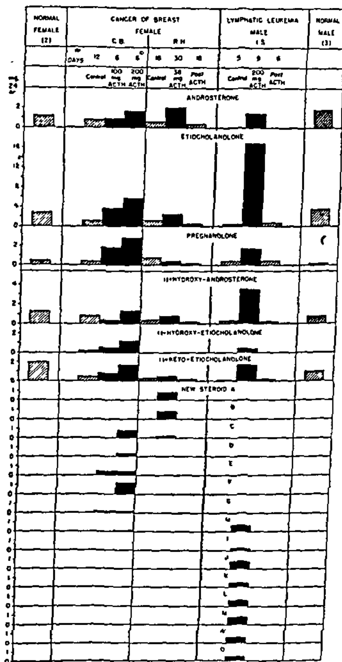


Fig. 6. Effect of ACTH administration on steroid excretion patterns in patients with neoplastic disease compared with the

adrenal metabolite, 11-hydroxyætiocholanolone, in a number of cancer patients before diagnosis of cancer indicates to me that there was already a metabolic change *before there was a cancer.*

FOLLEY: What about the possibility of excretion of steroids in the [illegible] danger of some erroneous conclusions?

DOBRINER. We have done one experiment on normal humans. We injected a labelled steroid, and worked up the fæces, and we could discover only microgram amounts of metabolites, while more than 70 per cent of the injected material was found in the urine in the first day. Different species have different routes of steroid excretion. Mice and rats excrete steroids in the fæces for the most part. The horse is supposed to excrete very little in the fæces, and cows excrete steroids mostly in fæces.

SOMMERVILLE. Dr. Pearlman finds many progesterone metabolites in the bile of cattle, but not in that of pregnant women Have you used both cortisone acetate and cortisone, and do you find any difference in the urinary steroid pattern?

DOBRINER. We have only used cortisone acetate

SOMMERVILLE. I ask because when I administered pregnanediol to human subjects it reappeared in the urine, whereas administered pregnanediol diacetate did not do so.

DOBRINER· For metabolic studies we would like to have the free steroid, but since we could get only the acetate, we have studied that derivative.

BOYLAND Is there any evidence that response to ACTH is dependent on the saturation with ascorbic acid, or do all patients have plenty of ascorbic acid?

[illegible] deficient diets There was considerable difficulty in getting the patients to take the diets, and when we got the riboflavin levels in the urine, it turned out that the diets weren't completely deficient.

[illegible] a powerful leukæmogenic agent?

BURCHENAL: So many of the compounds which have an effect on cancer are also carcinogenic. I don't know whether it would be a leukæmogenic agent or not.

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DISCUSSION

DOBRINER: We do feel that we are justified in speaking of adrenal hormonal deficiency in neoplastic patients in the same way that we speak of a conditioned vitamin deficiency. The adrenals in these patients may be functioning well enough to supply the hormones required by a normal person, but in disease the tissues require larger amounts of adrenal hormones. Under conditions where the tissues require a larger amount of hormones, in this case adrenal hormones, there seems to be some mechanism which we don't understand, causing cessation of stimulation of the pituitary gland.

BEGG: There are really two aspects to this question: these changes in adrenal steroid patterns previous to the detection of the tumour may be one aspect, and another aspect the changes that take place in a host after the tumour is there.

DOBRINER: I was always very careful not to make a statement of what was primary and secondary. But certainly the occurrence of this

CHAIRMAN'S CLOSING REMARKS

You will agree it is difficult or perhaps impossible to summarize a meeting of this kind, so that the following are simply a few cursory remarks. I am sorry Astbury is not here this afternoon because I found his reaction to the meeting, especially the biological aspects of the subject, intriguing. I myself have never been an endocrinologist, but have always looked on the endocrine side of this field as certainly one of the most complex and the particular steroid field as the most complex sector of it. Astbury was very disturbed and anxious, listening to the biologists in particular, wondering how it was that they could deal with so involved a situation, when in experimentally varying one factor, and perhaps that rather inadequately known, they might also be varying a whole congeries of still other factors, quite unknown. On the other hand, I don't think we need feel too anxious : complexity is an inherent quality of the subject, and the field must be attacked subject to all these limitations.

If the best approach is that of planned experiment, it has also to be combined with a certain biological acumen. It is certain that Gardner's contribution was a very fine example of the planned experiment, with quite specific alterations of experimental procedure, everything being controlled as well as possible, and, as we now find, beginning to yield something which has meaning. As for biological acumen, we also find that beautifully illustrated, combined with the planned experiment, in Huggins' work, of which we had such a wonderful account this morning.

If I may say so, I think it would be quite unwise to adopt [illegible]

the thing that crops up is very often some feature quite unexpected that the screen wasn't devised to detect, so that the

GARDNER: Did you notice any effect on alopecia and hair growth in your patients?

BURCHENAL: We have seen it in one patient, a girl treated with ACTH. She did not get a total alopecia as you see with the antifolics sometimes, but her hair definitely thinned out. However, on continued therapy, I think that wasn't so noticeable. I have not noticed it with the other ones. We have seen hirsutism in at least two children who were treated with cortisone. I don't think there was any other evidence of virilism.

BOYLAND: Does cortisone produce chromosome abnormalities? It would be interesting to see if it produces grey hair in dark mice.

DOBRINER: There are some experiments underway on that.

GARDNER: Baker has shown that if cortisone is topically applied it will prevent hair growth locally, and if systemically applied it will [illegible] same [illegible] rague [illegible] 'H.

HERTZ: Thorn found an increased pigmentation about the site of Compound E pellets.

BURCHENAL: One of our leukæmic patients, a boy of 16, developed a [illegible] see it often.

KELLIE: It is not very marked?

BURCHENAL: It depends. You don't push many patients to full Cushing's. I think if you did you would get it more often.

BEGG: The Mayo people have included the production of striæ as part of their characteristics of the side effects of cortisone therapy.

CHAIRMAN'S CLOSING REMARKS

You will agree it is difficult or perhaps impossible to summarize a meeting of this kind, so that the following are simply a few cursory remarks. I am sorry Astbury is not here this afternoon because I found his reaction to the meeting, especially the biological aspects of the subject, intriguing. I myself have never been an endocrinologist, but have always looked on the endocrine side of this field as certainly one of the most complex and the particular steroid field as the most complex sector of it. Astbury was very disturbed and anxious, listening to the biologists in particular, wondering how it was that they could deal with so involved a situation, when in experimentally varying one factor, and perhaps that rather inadequately known, they might also be varying a whole congeries of still other factors, quite unknown. On the other hand, I don't think we need feel too anxious : complexity is an inherent quality of the subject, and the field must be attacked subject to all these limitations.

If the best approach is that of planned experiment, it has also to be combined with a certain biological acumen. It is certain that Gardner's contribution was a very fine example of the planned experiment, with quite specific alterations of experimental procedure, everything being controlled as well as possible, and, as we now find, beginning to yield something which has meaning. As for biological acumen, we also find that beautifully illustrated, combined with the planned experiment, in Huggins' work, of which we had such a wonderful account this morning.

If I may say so, I think it would be quite unwise to adopt too physical or too rigid an approach to the problem of screening. Certainly have the screen planned, thought out, designed as much as possible, but our experience is that, in spite of all, the thing that crops up is very often some feature quite unexpected that the screen wasn't devised to detect, so that the

factor of acumen or whatever you may care to call it is undoubtedly important.

I think we had at this meeting preliminary signs at any rate of some linking between this field and others, an essential process if the eventual picture is to be real. We separate them for mental convenience, but it is encouraging to feel that a link is beginning to appear between this field and, say, the general field of protein chemistry in relation to cancer. We had hoped Sir Robert Robinson might be here, bearing specially in mind his stimulating notion that the administration of a fresh steroid may result in the appearance of specific proteins.

Another matter of some interest is that the tumours which have responded in the chemotherapy results described by Dr. Burchenal are approximately the same as those which react to other chemotherapeutic procedures. We have been very puzzled to know why the lymphomata, plasmacytomas, reticuloses and so forth respond so briskly to the mustards, for instance, and why other malignant cell types don't so react. *One possibility is that these are the tumours derived* from the normal cell types particularly concerned with the production of protein in high amount, and many of them with the production of immune globulins and so on. We have wondered whether chemotherapeutic agents may have a selective action on these cells because they are possibly the seat of highly active specific protein synthesis in a way that other cells perhaps are not.

Apart from the questions of protein and protein anabolism and catabolism and so forth, in Hertz's work again we see interesting beginnings of a link-up with the general field of cell nutrition. I said at the time that Gardner's paper seemed to throw out a general principle, or possibility of such, in the significance of gonadotrophin in carcinogenesis. We made an interesting observation in this field about two years *ago.* Actually the fact was noted without our being aware of the proper circumstances for some time, but it appears that in the growth of two or three rapidly growing rat tumours there

is a most remarkable atrophy of the seminal vesicle. In the meantime, we have tried to determine what are the governing conditions, and it has been suggested that gonadotrophin may, in fact, be significant here. We wonder whether, as well as in carcinogenesis, a similar requirement may be important in the growth of the tumours themselves.

Then again Dobriner has just given us another example of work of great complexity and detail, but with two threads running through, that malignant disease may be associated either in its inception or course with decreased gonadal and adrenal function, and that the therapeutic action of ACTH and cortisone may possibly be based on specific adrenal stimulation of one kind or another.

Finally, we all felt that Foulds' account of the behaviour of the mammary tumours he described, being so susceptible, so dependent, and showing a most remarkable regression at the time of parturition and at the beginning of lactation, is a matter which, as I think he realizes, should be followed out very vigorously.

Those of us from this country are specially grateful for the massive American contribution to this meeting. In conclusion, I should like to convey my personal thanks, and thanks also on behalf of the Foundation, to all the speakers for the time and care which they have so generously devoted to the aims and success of the Colloquium.

BOOK II

STEROID HORMONES AND ENZYMES

AT the time this colloquium was held, programme speakers were not asked to prepare or submit any manuscript. The editors are most grateful to the contributors for kindly providing *summaries* of their work, on which their remarks were based. The *general discussions* are, however, reproduced almost in full.

ASSAY, ACTIVITY AND PURIFICATION OF β-GLUCURONIDASE

W. H. FISHMAN

I. Methods of Assay

THE hydrolysis of menthol glucuronide to menthol and glucuronic acid by tissue β-glucuronidase was first employed as the basis for glucuronidase assay by Masamune (1933). Measurements were made of the increase in the reducing power of the digest which is due to the liberated aldehyde group of glucuronic acid. At present the determination of reducing power is performed with a modification of the ceric sulphate reductimetric method of Miller and Van Slyke (1936). The disadvantages of this method are: (1) the necessity of using rather long periods of incubation; and (2) the residual reducing power of the tissue extract may be high in crude extracts. Nevertheless, the reductimetric method for determining β-glucuronidase activity has yielded valuable information in studies on the purification of the enzyme and on the determination of reaction kinetics.

The method of measuring β-glucuronidase activity employing phenolphthalein β-glucuronide (Talalay, Fishman, and Huggins, 1946; Fishman, Springer and Brunetti, 1948) is based on the principle that phenolphthalein in alkaline solution absorbs light at 540 millimicrons to an extent five hundred times that of the absorption of its glucuronide. Accordingly, measurements are made of the optical density of the alkalinized digests under standard conditions. The method is termed an *aglucuronometric* method, since the principle of the determination depends on the properties of the aglucurone radical. The same principle has been used by

Kerr (Kerr, Graham and Levvy, 1948) and Mills (1948) in their use of phenyl glucuronide as a substrate for β-glucuronidase. In this method, the liberated phenol was measured by means of the Folin-Ciocalteu reagent. Apparently the enzyme extract requires purification in order to reduce the value of the phenolic substances in the control digest. At the present time, the majority of investigators in this field employ phenolphthalein glucuronide as the substrate with which to assay glucuronidase activity.

II. Factors in Blood Which Influence β-Glucuronidase *Activity*

A. The addition of plasma to purified β-glucuronidase.

It was found that the addition of plasma to purified dog liver β-glucuronidase in increasing amounts resulted in a progressive inhibition of the liver β-glucuronidase activity. This inhibitory activity is a property of a heat-stable, non-dialysable, protein-like component or components of the plasma. The inhibition appears to be a function of the enzyme concentration in the digest and does not influence the character of the *p*H-activity curve.

Studies have been done on plasma separated from the blood of human subjects with and without disease, in order to determine whether *any characteristic increase or decrease* of glucuronidase inhibitor was associated with the condition of the subject. In about 80 per cent of all subjects studied a progressive inhibition of purified β-glucuronidase is observed upon increasing the relative amount of plasma in the digest. Another 10 per cent of the patients did not show the presence of as much glucuronidase inhibitor as did the first group. In the remainder an interesting phenomenon was observed: at low concentration of the plasma in the digest, an activation rather than an inhibition of glucuronidase activity occurred, and with higher plasma concentration this effect may be reversed to one of inhibition. *No explanation can be advanced* for these findings at the present time.

B. Effect of dilution of the tissue homogenate on β-glucuronidase activity.

When one determines β-glucuronidase activity in different dilutions of the same homogenates of mouse tissues, essentially the same activity per gram of tissue is found. On the other hand, homogenates of rat and dog tissues often show higher activity per gram of tissue at low dilution of the homogenate. These findings emphasize the importance of working out systematically the best conditions for assay of β-glucuronidase in tissues of any given species. It was noted, also, that the tissues of the rat contained considerably more β-glucuronidase activity than did the tissues of the dog, mouse, or rabbit.

The observations described above made it clear that their adequate interpretation required a knowledge of the properties of pure β-glucuronidase. Once such a study had been made, the phenomena observed with crude extracts of liver glucuronidase could be reinvestigated.

III. Purification of β-Glucuronidase

The work which will now be described has been done in large part by Dr. Bernfeld in my laboratory. Many observations (Oshima, 1936; Karunairatnam and Levvy, 1949) indicated that a large variety of organic acids will inhibit the activity of β-glucuronidase. These include citric, malic, saccharic, gluconic, glucuronic, and ascorbic acids, as well as heparin. As a working hypothesis, we have assumed that β-glucuronidase in tissue may be combined with a number of acidic materials. Accordingly, in the purification procedure which has been developed, precipitation of the enzyme by ammonium sulphate in alkaline solution has been a new step which has been introduced. At the end of this purification procedure the preparation exhibits only one *p*H optimum at 4·5.

It has been reported by Mills (1948) and Levvy (Kerr, Graham and Levvy, 1948) that certain tissues contain several glucuronidases which differ only in their *p*H optima. When our best glucuronidase preparations are tested in the

presence of various acids such as adenylic, glucuronic, saccharic, and deoxyribonucleic acids separately, the pH optimum shifts to a higher value. Accordingly, it is suggested that the enzyme preparations of Mills and Levvy which show two peaks in the pH activity curve represent, not two different enzymes, but the same enzyme combined with one, two, or more different acidic compounds.

It was further observed that our purest β-glucuronidase preparation exhibited a marked activation of its activity in the presence of deoxyribonucleic acid. Accordingly, it is bel ... idase in tissue represents a complex of the ... one of the most important of ... e significance of this postulation remains to be established through more experimental work.

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DISCUSSION

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enzyme activity?

FISHMAN. We have studied the effect of varying the ... centration on the activity of glucuronidase. The conditions that w... used in the dilution experiments were those constituting optim... ... and there is no doubt in my mind that th...

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... relationship between β-glucuronida...

... on?

... elative activity of β-glucuronidase ... te concentration, the curve ascen...

to a maximum and then drops with excess substrate. This was found to be the case with three different substrates. Accordingly, the concentration of substrate selected in the enzyme assay corresponds with the optimal concentration observed in the substrate-activity curves.

FOLLEY: Have you tried to apply the equation of Lineweaver and Burk? They have evolved an equation which can be fitted to curves like yours, which show inhibition at high concentrations. From it two constants can be calculated, which would presumably correspond to the values you have found.

FISHMAN: We have not made those calculations.

MILLS: At what *p*H did you carry out the alkaline ammonium sulphate precipitation?

FISHMAN: At *p*H 8.

MILLS: We have observed progressive inactivation of glucuronidase at *p*H 8 and above. When you said that after alkaline precipitation you get only one electrophoretic component with isoelectric point at *p*H 7·5–8, I wondered whether you might be actually destroying preferentially one of the enzymes. About the shift in *p*H optimum which you find in the presence of saccharic acid and similar compounds, I think that can be explained, as I will try to show later, on the dif- [illegible]

[illegible]

magnesium?

FISHMAN: We haven't found any specific effects of the cations which would lead us to believe that one of these was essential for activity of the enzyme.

MILLS: We have tried various metals, calcium, zinc, magnesium, copper, and so on, as inhibitors and none of them had any significant effect.

ELSON: Have you tried any serum from cancer patients? You mention one carcinoma of the stomach from which there is apparently no dilution effect. That might indicate that in cancer patients there is probably an absence of inhibitors.

FISHMAN: These observations concern only tissue β-glucuronidase activity when determined in the cancerous and the surrounding tissue. This phenomenon where the tumour tissue enzyme activity is not affected by dilution, may or may not be related to the disease.

ELSON: I was wondering whether you have tried it in the blood plasma. You mentioned the plasma in surgical cases, where it doesn't

show this inhibitory activity. It might be that cancer patients in general don't show this.

FISHMAN: I don't believe that I can make a definite statement because you can observe all varieties of behaviour towards glucuronidase activity in the serum or plasma of cancer patients: inhibition, no change, and activation. We would like to obtain pure β-glucuronidase and test the effects on it of plasma, serum and tissue extracts. It is difficult to interpret the results with the relatively crude enzyme extract employed, because it may contain constituents which will influence the results.

STOREY: Does the glucuronidase prepared by alkaline ammonium sulphate precipitation behave in a homogeneous manner over a range of *p*H?

FISHMAN: As far as we know it does.

THE NATURE, PROPERTIES AND FUNCTION OF β-GLUCURONIDASE

G T. MILLS

A. The Preparation and Properties of β-Glucuronidase

During the past few years we have carried out work on the purification and properties of glucuronidase in order to learn more about its fundamental properties as an enzyme, before proceeding to a study of the physiological function of this enzyme. We have used spleen as the starting material for the preparation simply because it is the richest known source of glucuronidase, and we have used ox spleen since the material is readily available. The method used for the preparation of active enzyme extracts (Mills, 1948) is, very briefly, a defatting and dehydration of the tissue with acetone, followed by a water extraction and ammonium sulphate precipitation, with subsequent solution in water. The fractionation of these extracts has been carried out with ammonium sulphate using concentrations determined from salting out curves at various *p*H's (Mills, 1948). More recently we have been experimenting with low temperature acetone methods of fractionation, and these are yielding promising results.

Our main finding is that in ox spleen there appear to be three glucuronidase activities. These three main activities are distinguished by different *p*H optima, different affinities for various substrates and differing behaviours towards various inhibitors.

Some of the characteristics of these three enzymic activities are recorded in Table I.

The glucuronidases are inhibited by many mono- and dicarboxylic acids, the most potent of which is D-saccharic acid as first shown by Karunairatnam and Levvy (1949). These inhibitors affect the glucuronidase in different ways

Table I

Properties of Ox Spleen β-Glucuronidase

Substrate	*Ox Spleen Glucuronidase*		
	I	II	III
pH optima (in acetate buffers)			
1-menthylglucuronide	4·5	5·0	3·4
phenylglucuronide	4·5	5·2	3·4
phenolphthalein glucuronide	4·5	5·2	3·4
Enzyme-substrates Dissociation constants at 38°C (calculated according to Lineweaver and Burk (1934))			
1-menthylglucuronide	$4{\cdot}7 \times 10^{-3}$	$19{\cdot}0 \times 10^{-3}$	
phenylglucuronide	$2{\cdot}0 \times 10^{-3}$	$5{\cdot}1 \times 10^{-3}$	$1{\cdot}2 \times 10^{-3}$
phenolphthalein glucuronide	$0{\cdot}8 \times 10^{-3}$	$2{\cdot}3 \times 10^{-3}$	$1{\cdot}0 \times 10^{-3}$
Energies of activation (calories/mol.)			
phenylglucuronide	14,700	16,000	19,300
phenolphthalein glucuronide	14,400	16,700	18,900

and some of the findings on the type of inhibition exerted are shown in Table II.

Many other inhibitors are known for glucuronidase, for example, ascorbic acid, as first shown by Becker and Friedenwald (1949); this substance inhibits all three enzymes non-competitively. The poly-sulphonic acid trypanocidal

Table II

Type of Inhibition of the Three Ox Spleen β-Glucuronidases Exerted by Certain Inhibitors

Inhibitor	*Enzyme*		
	I	II	III
Citrate	Competitive	No Inhibition	Competitive
Oxalate	*	No Inhibition	Competitive
Mucate	Competitive	Non-Competitive	Competitive
Saccharate	Competitive	Non-Competitive	Competitive

*Inhibition of such a low order as to prevent an accurate assessment of type.

drug Suramin inhibits glucuronidases at *p*H's below 4·5, and then shows no inhibition and even activation above *p*H 5·5. Heparin also inhibits the glucuronidases in a similar manner to Suramin.

From all the inhibition experiments it would appear that a prime requisite for a compound to inhibit glucuronidase is the possession of an acidic grouping in its molecule, which suggests that the active centre in the various glucuronidases may be basic in character.

An important question which arises concerning these three ox spleen glucuronidases is one which must be posed whenever an enzymic activity is fractionated into a number of apparently separate entities. Are we dealing with separate entities existing as such in tissues, do we modify one single enzyme in various ways during fractionation by the partial removal of inhibitory or activating substances, or do we start with a parent molecule and break it up into sub-units, each having distinctive properties? Evidence which bears on the problem includes the *p*H activity curves of an ox spleen extract at various substrate concentrations.

We have found that by carrying out such *p*H activity curves at different substrate concentrations in the range 0·00025 to 0 004 M phenolphthalein glucuronide, we obtain curves of widely differing shapes with optima at the various points which may be predicted from the behaviour of the purified fraction at various substrate concentrations, and we feel that this is good evidence that we are not simply producing artefacts by the fractionation procedures used.

Evidence from adsorption and electrophoretic studies of glucuronidase leads us to believe that glucuronidase exists as a complex of closely related proteins in the original tissue, possibly a series of protein molecules on a carrier protein molecule and that each activity can be separated, but is only active in the presence of the carrier molecule.

From our electrophoretic and other studies, we have concluded that the isoelectric points of the various glucuronidases all lie near to *p*H 5.

Table I

PROPERTIES OF OX SPLEEN β-GLUCURONIDASE

Substrate	Ox Spleen Glucuronidase		
	I	II	III
pH optima (in acetate buffers)			
1-menthylglucuronide	4·5	5 0	3·4
phenylglucuronide	4 5	5·2	3·4
phenolphthalein glucuronide	4 5	5·2	3·4
Enzyme-substrates Dissociation constants at 38°C (calculated according to Lineweaver and Burk (1934))			
1-menthylglucuronide	$4{\cdot}7\times10^{-3}$	$19{\cdot}0\times10^{-3}$	
phenylglucuronide	$2{\cdot}0\times10^{-4}$	$5{\cdot}1\times10^{-4}$	$1{\cdot}2\times10^{-3}$
phenolphthalein glucuronide	$0{\cdot}8\times10^{-3}$	$2{\cdot}3\times10^{-4}$	$1\ 0\times10^{-4}$
Energies of activation (calories/mol)			
phenylglucuronide	14,700	16,000	19,300
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Many other inhibitors are known for glucuronidase, for example, ascorbic acid, as first shown by Becker and Friedenwald (1949); this substance inhibits all three enzymes non-competitively. The poly-sulphonic acid trypanocidal

Table II

TYPE OF INHIBITION OF THE THREE OX SPLEEN β-GLUCURONIDASES EXERTED BY CERTAIN INHIBITORS

Inhibitor	Enzyme		
	I	II	III
Citrate	Competitive	No Inhibition	Competitive
Oxalate	*	No Inhibition	Competitive
Mucate	Competitive	Non-Competitive	Competitive
Saccharate	Competitive	Non-Competitive	Competitive

*Inhibition of such a low order as to prevent an accurate assessment of type.

level by 20 days of age, and is thereafter constant. From our results it is clear that the glucuronidase activity in the liver is constant when the liver is increasing at its most rapid rate, and this would not indicate any connection between glucuronidase activity of the liver and the growth rate of the liver. What this fundamental change in the glucuronidase content of the cell at 20 days of age means is not yet clear. It may be coincidental or not that at this age the animals are weaned, and the change in glucuronidase may be related to nutritional factors or to a change in the state of the endocrine balance of the animal.

We have found that the glucuronidase activities of the liver determined at the four *p*H values, 3·4, 4 5, 5·2, and 7·0, behave in an identical manner, and we take this to mean that all the glucuronidases of the liver behave as a group during the growth and maturation of the animal. We have found the same to apply in all other experiments we have carried out on the glucuronidase activity of the liver in various conditions.

We next turned to a study of rat liver during its phase of rapid growth which follows partial hepatectomy. Levvy *et al.* (1948) recorded that in mouse liver tissue following partial hepatectomy, there was an increase in glucuronidase activity 3–8 days after operation. In our experiments approximately 70 per cent of the liver of male albino rats was removed by the method of Higgins and Anderson (1931); the remaining 30 per cent rapidly regenerates, reaching 70 per cent of normal weight in 4 days and 100 per cent in 15 days.

Our results show that there is no change in liver glucuronidase activity during the first three or four days after operation, when liver weight, liver protein and liver DNA are increasing most rapidly, and the increase in glucuronidase activity only occurs when the rapid phase of growth is over.

This finding suggests that the increase in glucuronidase activity of rat liver which occurs 4 days after partial hepatectomy and which persists for up to 20 days, must be related to some other factor than proliferation. We have reached the

We have recently turned to a study of the glucuronidases of other tissues, particularly the liver. In this organ there definitely appears to be another glucuronidase, with optimal activity around pH 6·5–7·0. We had already gained the impression from kinetic examinations of ox spleen extracts, that such an enzyme existed in spleen, but in liver this activity at pH 6·5–7·0 is much more obvious. We are at present studying the separation of the liver glucuronidases by ammonium sulphate fractionation and low temperature acetone fractionation, and have obtained fractions containing increased amounts of this fourth glucuronidase.

In all experiments on the physiological function of glucuronidase we have assayed tissue extracts at four pH's, namely 3·4, 4·5, 5·2, and 7·0.

B. The Function of β-Glucuronidase

The function of glucuronidase in tissues is still a matter of conjecture, but various theories have been advanced concerning its mode of action. There is the metabolic role suggested by Dr. W. H. Fishman (Fishman, 1940, 1947; Fishman and Anlyan, 1947), who will no doubt say more about this theory, and I will therefore make no further reference to it. Levvy and his colleagues (Levvy, Kerr, and Campbell, 1948; Kerr, Campbell and Levvy, 1949, 1950) have suggested a relationship between the glucuronidase activity of mouse tissues and the extent of cellular proliferation occurring in those tissues.

Most of our experiments have been carried out on rats, and, in the first place, we found that the liver glucuronidase level of embryonic and young rats was less than that of adult animals—the adult activity being reached at about 30 days of age.

We have employed the deoxyribonucleic acid (DNA) content of the tissues as an indication of cell number, as suggested by Davidson and Leslie (1950 *a* and *b*), and this enables us to obtain an indication of the glucuronidase activity per cell. Our results indicate that in the young rat the glucuronidase activity per cell is low at birth and rises to a constant

when œstrogen is first secreted by the ovary in sufficient amount to affect this very sensitive target organ. It is possible that these results could be related to yours.

Mills: Yes, the change in our experiments may be related to the endocrine state of the animals.

Fishman. I would like to mention a difference which we have observed in the behaviour of our purified β-glucuronidase in the presence of citrate. The enzyme after alkaline ammonium sulphate is activated by citrate ions.

In the course of our purification we have compared at each step the ratio of hydrolysis of phenolphthalein glucuronic acid and of menthol glucuronic acid, and have observed a constant ratio in the course of purification. This agrees with your observations, emphasizing the fact that there is no actual qualitative difference in the ability of the various

of 6 4–6·5

Mills: There is one point about the citrate activation mentioned by Dr. Fishman. We observed rather a peculiar result when endeavouring to determine the type of inhibition exerted by oxalic acid. It inhibited the glucuronidase at a low substrate concentration; as the substrate concentration increased, inhibition decreased, and at higher substrate concentrations the effect of the oxalate was reversed and became very slight activation, the activation effects being not more than 10–15 per cent. It appeared to be a function of the substrate concentration.

Fison: Do these differences with age

Fishman: The distribution of β-glucuronidase in mammalian tissue is very unusual, in that I have yet to find a tissue which did not contain some β-glucuronidase activity. This contrasts markedly with the

same conclusion from other experiments on liver damage caused by carbon tetrachloride which cannot be given in detail owing to lack of time. We have confirmed some of the experimental findings of Levvy and his colleagues on mice, but in view of our experiences with rats, we feel that the observed variations in liver glucuronidase activity must be related to some factor or factors other than cell proliferation, in all probability a similar process in both rats and mice.

An elucidation of the true function of β-glucuronidase will only be arrived at by the production of much more experimental evidence than we have at present available.

In conclusion I wish to thank Dr. J. Paul and Miss E. E. B. Smith for some of the results presented here.

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DISCUSSION

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ON THE MECHANISM OF SYNTHESIS OF CONJUGATED GLUCURONIDES

I. D. E. STOREY

The mechanism by which glucuronides are synthesized is of considerable interest in connection with the subject of this symposium, in view of the finding that certain of the steroid hormones and related compounds are excreted in this form by the animal body. It may be well to point out, however, that, besides its presence in these glucuronides of relatively low molecular weight, glucuronic acid is widely distributed throughout the body in mucopolysaccharides such as chondroitin sulphate and hyaluronic acid, and it is therefore a substance of great physiological importance.

In the early work on the origin of glucuronic acid and of glucuronides, in most cases the intact animal was used, and interpretation of the results is generally difficult. Suffice it [illegible] cursor. As such, he suggested lactate, or some other 3-carbon compound derived from the breakdown of glycogen. Similar conclusions were reached by Lipschitz and Bueding (1939) in their experiments with tissue slices. Using livers from fasted guinea-pigs, and borneol or menthol as glucuronidogenic agents, they were unable to demonstrate appreciable glucuronide formation from hexoses, glucosides or from glucuronic acid itself, but striking increases were obtained when lactate, pyruvate or dihydroxyacetone were added to the medium. Synthesis did not take place anaerobically, and cyanide, fluoride and iodoacetate all inhibited the process. From these results, they concluded that the synthesis was dependent upon oxidative processes as the source of energy, and that phosphorylations were involved.

situation for pepsin or trypsin, or even the acid and alkaline phosphatases. In order to construct a picture of the function of this enzyme, one must take into account the comparatively wide distribution of the enzyme in the tissue.

MEYER: Do you find a constant ratio of these four enzymes in different batches of spleen?

MILLS: No, with spleen we don't find very much of the *p*H 7 enzyme present at all. With the other enzymes we do find variations in the shape of the crude mixture *p*H activity curve. There do appear to be sometimes slight variations from one batch to another. They are not really fundamental changes. It may be just the way the spleens are treated before we get them.

MEYER: So the relationship between activities of each of those is constant from batch to batch?

MILLS: Relatively constant.

iver slices) it suppresses glucuronide synthesis at least 90 per cent.

Sulphate ion is also an inhibitor of the synthesis, and this has been shown to be due to the formation of an ester sulphate of o-aminophenol. It thus appears that the ester sulphate synthesizing system is competing with that forming glucuronide for the *o*-aminophenol available.

In the present work, the only substance which has been observed to cause any stimulation of the synthesis of glucuronides is bicarbonate. The rate of synthesis in phosphate Ringer is very low, but the addition of as little as 2·7 millimols /litre of bicarbonate can double the rate, and Krebs's bicarbonate Ringer, which contains about 16 millimols/litre at *p*H 7·3, raises it still further. This effect of bicarbonate is independent of the nutritional status of the animal, and is not due to changes in the *p*H of the medium, or to differences in respiratory rates in the two types of media. The most probable explanation appears to be that carbon dioxide fixation is in some way concerned in glucuronide synthesis. In an attempt to elucidate whether the Woods-Werkman reaction (carboxylation of pyruvate) or the carboxylation of α-ketoglutarate might be involved, experiments were performed involving the addition of certain components of the tricarboxylic acid cycle, such as succinate and malate, to slices in phosphate Ringer, but the results were inconclusive. The small increases sometimes observed could well be explained as being caused by respiratory carbon dioxide.

Finally, it might be of interest to mention the relationship between glucuronic acid and certain phases of pentose metabolism. In pentosurics, when a glucuronidogenic drug is administered, a small amount of glucuronic acid may be excreted, but there is also a greatly increased excretion of pentose (L-xylulose). Furthermore, Enklewitz and Lasker (1935) have shown that pentosurics give an increased excretion of pentose after administration of glucuronic acid. It would seem that these observations are well worthy of further investigation.

This *in vitro* work has been continued in the present investigations (Storey, 1950), but a simpler and more rapid method for the measurement of glucuronide synthesis, using o-aminophenol as aglycone, was employed (Levvy and Storey, 1949). With both fasted mice and guinea-pigs, considerable conjugation was observed even in the absence of the above-mentioned 3-carbon compounds, and addition of these to the medium at a concentration of 0·02 M was without effect. The reason for the discrepancy between these results and those of Lipschitz and Bueding is not at present apparent. Furthermore, the last-named authors used glucuronate at a concentration of only 0·005 M, whereas it has been found that at 0·02 M, it inhibits glucuronide synthesis 85 per cent, and at 0·01 M, 56 per cent. Gluconate and saccharate are also marked inhibitors, 26 per cent and 30 per cent respectively at the lower concentration, whereas all the other monocarboxylic and dicarboxylic acids tested showed only slight inhibitory activity. This apparently specific effect of glucuronate suggests that an analogy might be drawn with the catalysis by phosphorylases of glycosidic linkage formation, to form starch, glycogen and nucleosides, in all of which an aldose-1-phosphate is one component of the system. Cori, Cori and Green (1943) showed that glucose competitively inhibits the formation of glycogen by liver phosphorylase, and in the present instance it seems a reasonable explanation that glucuronate may be competing with glucuronic acid-1-phosphate, or some closely related compound.

Evidence has been obtained that glucuronide synthesis, like other biochemical synthetic processes, depends on high energy phosphate generated by oxidative metabolism. The observations of Lipschitz and Bueding (1939) on the inhibitory effect of cyanide have been confirmed, but their experiments with fluoride and iodoacetate are difficult to interpret. One agent which is known to suppress the formation of high energy phosphate is 2:4-dinitrophenol, and in the present work, at a concentration of 1×10^{-4} M (which may actually increase the rate of respiration of

investigate systems in which the aglycone is an alcohol which may not conjugate with sulphate.

STOREY: Regarding the relative rates of synthesis, the rates of synthesis which we have observed with *o*-aminophenol are approximately the same as those observed by Lipschitz and Bueding using

isolation.

FISHMAN. With regard
vitro, the relative failure
selves to observe incorpo

synthesis

MILLS: This is very interesting
acid is
gates of
acid is
that the
It is quite possible that the synthetic system and conjugation system may be quite distinct, and that glucuronidase may play some part in

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DISCUSSION

... the work of Lipschitz and Bueding it is of

... never been reported in nature.

I also found it difficult to interpret Lipschitz and Bueding's data because they made no distinction between the processes of glucuronic ... and the ... to ...

... glucuronate were more marked than those of saccharate, citrate and gluconate. We have just seen data that these substances are all inhibitors for the hydrolytic activity of β-glucuronidase in extracts, and we now see that they are inhibitors of synthesis. The failure to observe conjugation when pure glucuronic acid is present in the system may not be surprising. Lipschitz and ...

... er unusual ... tive amine which may undergo reactions in the *in vitro* system, and I would think it would be important to isolate and identify aminophenylglucuronide after the slices have presumably synthesized a large quantity of this ...

... ation and glucuronide ... Sulphates and glucu- ... We probably should

THE METABOLISM AND EXCRETION OF SYNTHETIC ŒSTROGENS, WITH SPECIAL REFERENCE TO THE FORMATION OF THE GLYCURONIDES

D. H. CURNOW and E. C. DODDS

THE two most well-known mechanisms employed by the animal body for the detoxication and elimination of phenols are glycuronide and ethereal sulphate formation. It has long been the practice in carrying out experiments on the metabolism of phenols to measure increases in the total combined glucuronic acid excreted, or increases in the total ethereal sulphate, produced by feeding or injecting the phenol under study. In view of the large normal daily variation in the excretion of glucuronide in the urine, it is only possible to use these methods when large doses of phenols are administered. With the therapeutic dose of stilbœstrol, from a fraction of a milligram to 50 mg. daily, it is necessary to follow its conversion to glucuronide by some other method. Much larger doses, however, may be given to experimental animals, and the possibility of isolation of the metabolic products is greatly increased.

Although other workers have studied the conversion of some of the synthetic œstrogens to glucuronides, and Mazur and Schorr isolated stilbœstrol monoglucuronide from the urine of stilbœstrol-treated rabbits, it is proposed here to follow more closely the series of researches carried out at the Courtauld Institute of Biochemistry under the direction of Professor Dodds.

It had been shown that the urinary excretion of administered synthetic œstrogen in a biologically active state was much higher than in the case of administered natural steroid œstrogens.

STOREY: If you leave the magnesium out of the medium, it doesn't make much difference.

FOLLEY: Would it have the effect of decreasing the calcium ions? Have you investigated the effects of concentration of calcium on the synthesis?

STOREY: Calcium in the medium has no effect.

WILLIAMS-ASHMAN: Have you tried this work in homogenates?

methylene blue and brilliant cresyl blue on glucuronide synthesis? They also will uncouple oxidation from phosphorylation.

STOREY: I haven't done that. The dinitrophenol effect is rather nice and I thought the dyes would not penetrate.

WILLIAMS-ASHMAN: Methylene blue would.

STOREY: Also we are up against difficulty in the way of interference in the colour reaction. *ortho*-Aminophenol is not the ideal substance, but it gives a very rapid and very easy way of estimating activity. There is also the great advantage that you can estimate glucuronide directly instead of by the disappearance of your phenol.

sulphate and glucuronide formation. The ethereal sulphate is preferentially formed in a number of compounds, particularly ones which do not contain a phenol group; but with certain compounds, e.g., 4-aminodiphenyl, the aminostilbenes, and benzidine, the ethereal sulphate mechanism seems to fail, and they are excreted as glucuronides. We were struck by the fact that this process seems to be associated with increased toxicity and even with carcinogenicity, particularly in the case of the aminostilbenes.

*We have recently reported glucuronide synthesis by liver homogenates (Dutton, G. J., and Storey, I. D. E., 1951, *Biochem. J.*, 48, xxix). The product

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Stroud, at the Courtauld Institute, injected the synthetic œstrogens, hexœstrol, dienœstrol and stilbœstrol subcutaneously into rabbits, and studied the recovery in the urine of œstrogenically active material, by bioassay. His treatment of the urine consisted of a 20 hr. continuous extraction with benzene to obtain the "free" œstrogen, followed by 2 hours acid hydrolysis of the residual urine, and another 20 hr. benzene extraction to recover the "combined" œstrogen. In this way he obtained from 17–25 per cent of injected hexœstrol and stilbœstrol and 7 per cent of the injected dienœstrol. The reason for the low figure for dienœstrol will be seen later. Of the excreted œstrogen, some 70 per cent was found in the "free" fraction and 30 per cent "combined." These figures were obtained by *bioassay*, although some 5–15 per cent was recovered, after hydrolysis, as the crystalline hormone.

On the other hand, œstrogenic activity from the urine of *œstrone*-treated rabbits accounted for only 1·5 per cent of the activity of the injected hormone, 0·5 per cent "free" and 1 per cent "combined." From the urine of rabbits injected with 1·5 mg. of œstrone, 8 mg. of œstrone and 16 mg. of, probably, β-œstradiol were isolated. These experiments suggested a difference in the metabolic paths between the synthetic and the natural œstrogens.

To obtain more information on the metabolism of the synthetic œstrogens, Stroud next investigated the metabolism of some of the parent hydrocarbons of the œstrogens, and found that they were excreted as the corresponding phenols, e.g., diphenyl was converted to 4-hydroxydiphenyl in 25 per cent yield, stilbene to 4:4′-dihydroxystilbene in 3 per cent yield. No trace of the original hydrocarbons was found in the urine. The metabolism of diphenyl ether, which is itself not œstrogenic, is interesting in that it is excreted as 4-hydroxydiphenyl ether, which is an œstrogen, although a very weak one—100 mg. being required to produce vaginal œstrus in ovariectomized rats.

Continuing this type of approach, the metabolism of 4:4′-dimethoxydiphenyl ether and of 4-methoxydiphenyl was

investigated. In the rabbit the dimethoxydiphenyl ether was demethylated to 4-methoxy-4′-hydroxyphenyl ether and the mono-methoxydiphenyl to 4-hydroxydiphenyl; a little 4:4′-dihydroxydiphenyl was also produced.

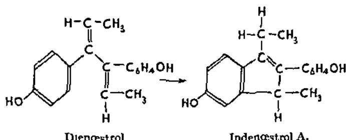
This work was followed up by Wilder Smith, Williams and Mrs. Simpson from a rather different point of view. In the treatment of cancer of the prostate with œstrogens the effective control of the carcinoma begins to fail after some time. It was thought that some adaptation by the body, involving changes in the metabolism of the œstrogen, may be responsible for the failure of œstrogen therapy.

The methods of study of urine from œstrogen-treated animals and man are, briefly, these: The urine is acidified with dilute HCl and extracted with ether. This ether extract contains the free phenols, and the glucuronides. These are separated by extracting the glucuronides as the sodium salts with saturated sodium bicarbonate. The phenols are then removed from the ether by NaOH extraction. The original urine containing any ethereal sulphate is hydrolysed and the liberated œstrogenic phenol extracted with ether. The three fractions—free phenol, glucuronide and sulphate—are then assayed in ovariectomized rats, or in the case of the glycuronide, may be assayed gravimetrically.

By this means it was shown that in rabbits injected with the synthetic œstrogen, up to 27 per cent of injected dienœstrol was eliminated as the glucuronide, 1–5 per cent appearing as the free phenol. With stilbœstrol the figure was sometimes as high as 46 per cent of the injected dose recovered as the glucuronide and up to 15 per cent as the free phenol.

In the cat, on the other hand, there was a much lower excretion—4 per cent recovery as glucuronide and 1 per cent as free œstrogen. The urine of the cat is acidic, while that of the rabbit is alkaline under normal conditions, but this difference was shown not to be responsible for the low excretion of glucuronide in the cat, in that producing acid rabbit urine by dietary means did not prevent the usual large excretion of œstrogen glucuronides.

In these experiments it was found that acid hydrolysis of urines containing dienœstrol always caused a loss of about 90 per cent of the original activity. This was traced to a facile cyclization of dienœstrol under acid conditions to produce the corresponding indene, identical with Hobday and Short's "*iso*dienœstrol" and Adler and Hagglund's "indenœstrol A."



Dienœstrol Indenœstrol A.

This compound possesses about one tenth of the activity of dienœstrol, and it is this which probably accounts for the low recoveries of dienœstrol obtained by Stroud in his early work.

But to return to Wilder Smith's work—very similar results to those obtained with *injected* œstrogens were obtained after the *oral* administration of hexœstrol, dienœstrol or stilbœstrol to rabbits.

Orally administered stilbœstrol glucuronide was excreted as the glucuronide in 13 per cent yield; subcutaneous injection produced a 25 per cent excretion of the same substance. This appears to show that either the glucuronide is not the end point of œstrogen metabolism or that the glucuronide is hydrolysed, some of the œstrogen takes other routes of metabolism and the usual 25 per cent or so is excreted again as the glucuronide.

In prostatic cancer patients, administered stilbœstrol is excreted in the urine as the glucuronide in yields of 10–60 per cent, and in normal women 10–75 per cent of administered [illegible] is recovered as the glucuronide. In the prostate

patients up to 9 per cent was excreted as the ethereal sulphate, and in the normal women up to 5 per cent. In the rabbit there was a much lower excretion of sulphate, as little as 0·04–0·5 per cent of injected stilbœstrol, hexœstrol or dienœstrol appearing in this form. There is no evidence that prostatic cancer patients excrete excessively large amounts of the glucuronide, and some other factor must account for the failure of œstrogen therapy.

In the studies with rabbits, the monoglucuronides of stilbœstrol, hexœstrol and dienœstrol were isolated and identified and their chemical and biological properties were studied. They each have only 5–10 per cent of the œstrogenic activity of the free œstrogens, using either subcutaneous or intravaginal application in the bioassays.

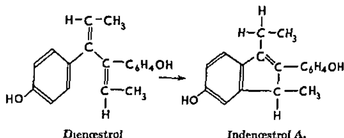
Recently we have started some work on the metabolism of 17-ethinylœstradiol. In the clinical use of this œstrogen there have been reports of sustained activity, and although these have not been very well supported, there has been suggested a stability in this compound not found with the unmodified natural œstrogens. Rabbits treated with ethinylœstradiol, however, do not excrete more than very small amounts of active material in the urine. This study is being pursued at the Courtauld Institute and should reveal some very interesting facts, especially with the use of a radioactive ethinylœstradiol.

In conclusion it is only necessary to re-emphasize the very important part that glycuronide synthesis plays in the metabolism and excretion of the synthetic œstrogens, and the ease of this synthesis and excretion, in contradistinction to the small excretion of the glycuronides of the natural steroid œstrogenic phenols.

DISCUSSION

[illegible]

In these experiments it was found that acid hydrolysis of urines containing dienœstrol always caused a loss of about 90 per cent of the original activity. This was traced to a facile cyclization of dienœstrol *under acid conditions to produce* the corresponding indene, identical with Hobday and Short's "*iso*dienœstrol" and Adler and Hägglund's "indenœstrol A."



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FOLLEY: I believe Emmenin, a preparation of œstriol glucuronide made in Montreal, is orally active. Does it give satisfactory results?

CURNOW: I don't know its activity relative to œstriol.

FOLLEY: Have you any evidence of the occurrence of glucuronides in clover or grass?

CURNOW: [illegible]

grass.

no acti

[illegible]

pared by Dr. Graham, which I tried to use for pregnanediol glucuronide in urine, but the hydrolysis was far from complete.

FISHMAN: Dr. Dobriner standardizes his hydrolysis with pregnanediol glucuronide and gauges the amount of enzyme to use from its [illegible]

[illegible]

we have needed rather a lot of spleen to get enough enzyme.

MILLS Very roughly we get 3–5 mg. of phenylglucuronide hydro- [illegible]

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practicable, as seems to have been done.

FISHMAN. It has the advantage that there are no tarry products produced in the course of enzyme hydrolysis, in contrast to the material resulting from acid hydrolysis.

STOREY: How long do you have to incubate the urine with glucuronidase?

FISHMAN· I think that Dobriner incubates overnight, about 24 hours

STOREY: I wondered whether the ether extraction of the urine before doing the quantitative determinations on glucuronides is really foolproof Some glucuronides are not soluble in ether, that's why we do them in acid solution

CURNOW. We have tested the glucuronides of the synthetic œstrogens by adding them to urine, and extracting with ether. They were quantitatively recovered.

FOLLEY· Do you get a monoglucuronide of stilbœstrol?

glucuronidase. Would it be possible to use vegetable glucuronidases, like baikalinase or ones produced by micro-organisms?

FISHMAN: Dr. Dobriner at the Cancer Memorial Hospital is using spleen glucuronidase added directly to urine concentrates after they've been fractionated, and he is satisfied with the results of hydrolysis, in that apparently there are no artefacts produced, no changes such as occur with acid hydrolysis. Dr. Doisy and his group in St. Louis are

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administered.

CURNOW: With injected stilbœstrol glucuronide, although there is about 75 per cent of it not excreted as such, it is interesting that during

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mechanism.

deal.

THE EFFECTS OF HORMONES ON β-GLUCURONIDASE ACTIVITY

W. H. FISHMAN

Mouse Tissues

In 1940 it was observed (Fishman, 1940) that the β-glucuronidase activity of liver, kidney, and spleen of animals fed menthol or borneol showed an increase which was not seen in the sex organs. Later it was found (Fishman and Fishman, 1944; Fishman, 1947*a*) that upon ovariectomy of female mice, the uterine β-glucuronidase activity exhibited a decrease and that this activity could be restored to normal levels by the administration of œstrogen The enzyme activity of the non-sex organs, liver, kidney, and spleen, showed no significant change throughout these experiments. This effect seemed to be specific for œstrogens, and these observations have been confirmed and extended by ourselves and other investigators.

A fundamental question which presented itself for study was whether or not the increase in the glucuronidase activity of the œstrogen-stimulated uterus was the reflection merely of the growth of the tissue. When the data were examined, it was found that there was no correlation between the total uterine nitrogen and glucuronidase concentration. Since this study, we have encountered a number of situations in which marked growth of a tissue was not accompanied by an increase in its glucuronidase concentration; and conversely, tissues have been made to increase their glucuronidase activity markedly without any appreciable amount of cellular proliferation.

Several possibilities have been considered to explain the *in vivo* function of β-glucuronidase: the enzyme may be entirely hydrolytic, completely synthetic, both hydrolytic and

CURNOW: Yes.

FOLLEY: Why isn't it a diglucuronide? When one of the phenolic [illegible]

[illegible] for example), and there may be some diglucuronide present, although this is doubtful.

MILLS: Phenolphthalein glucuronide is also a monoglucuronide.

McDonald and Odell (1947), who limited their investigation to the serum β-glucuronidase activity. It is interesting to note that the elevation in β-glucuronidase activity of the serum and plasma coincides with the excretion of progressively greater amounts of conjugated steroid glucuronides in the urine.

It has been observed that the postpartum fall in serum β-glucuronidase activity could be postponed by the administration of stilbœstrol (Fishman, Odell, Gill and Christensen, 1950). This would suggest that œstrogens may be responsible, in large part, for the elevated serum β-glucuronidase activity seen at pregnancy.

Odell and McDonald (1948) also demonstrated that women with the syndrome of pre-eclamptic toxæmia often possessed extraordinarily high serum β-glucuronidase values.

Observations on Human Vaginal Fluid β-Glucuronidase Activity

Odell and co-workers have reported (Odell and Burt, 1949) that high glucuronidase activity in the vaginal fluid was characteristic of women with cervical cancer, and this characteristic could be used as a diagnostic aid. In our studies (Fishman, Kasdon and Homburger, 1950) we have concentrated, first, on investigating the influence of physiological factors on vaginal fluid β-glucuronidase activity. It was observed that in normal subjects a very wide range of glucuronidase activity in vaginal fluid occurred, with low values predominating in the pre-menopausal state and high values frequently occurring in post-menopausal women. It was also observed that in women who have undergone pan-hysterectomy in which there remains no uterine tissue, high values of vaginal fluid β-glucuronidase activity were frequently found. From this control study it was concluded that the *uncritical* determination of vaginal fluid β-glucuronidase activity cannot be used as a diagnostic screening procedure.

synthetic, or have another function still unknown. The physiological experiments dealing with tissue changes in β-glucuronidase activity have been interpreted on the basis that β-glucuronidase participates predominently in the synthesis of glucuronides, a process of "metabolic conjugation" in which the conjugate represents the form in which the hormone is utilized in the tissue.

Interaction of β-Glucuronidase and Œstriol Glucuronide *in Vitro*

From an examination of the Michaelis Constants (K_m) and the affinities $\left(\frac{1}{K_m}\right)$ determined in experiments (Fishman, 1939; Talalay, Fishman and Huggins, 1946) in which the various glucuronides were hydrolysed by spleen β-glucuronidase, it is evident that the enzyme has a rather high affinity for œstriol glucuronide. No significant difference was found in the K_m values obtained when œstriol glucuronide was hydrolysed by mouse ovarian and splenic glucuronidase preparations.

Concerning the *in vivo* function of β-glucuronidase, it is desirable to point out that glucuronidase represents a protein of tissue which has a high affinity for œstriol glucuronide.

Blood β-Glucuronidase in Pregnancy and Toxæmias of Pregnancy

The activity of β-glucuronidase in the blood cells and plasma of women throughout pregnancy and parturition (Fishman, 1947*b*) has shown a relatively high concentration of the enzyme in the cells (which is true also of non-pregnant subjects) and a progressive increase in the glucuronidase which is terminated at parturition. This phenomenon has been investigated extensively and independently by

McDonald and Odell (1947), who limited their investigation to the serum β-glucuronidase activity. It is interesting to note that the elevation in β-glucuronidase activity of the serum and plasma coincides with the excretion of progressively greater amounts of conjugated steroid glucuronides in the urine.

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The level of β-glucuronidase activity in the vaginal fluid in menstruating women appears to be under the control of ovarian function. Thus, we have found a low range of values at mid-menstrual cycle. At the present time, work is under way in an attempt to further define the factors which control vaginal fluid β-glucuronidase activity, so that it may be possible to utilize this phenomenon for clinical purposes.

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DISCUSSION

FOLLEY: What do you understand exactly by vaginal secretion?

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of

FOLLEY:

cervical secretion.

FISHMAN: We know little concerning the glucuronidase activity of

They produce

when you can pull it out in long threads, sometimes as much as one or two feet long, whereas at mid-cycle it is sticky and won't pull out in

the changes in glucuronidase

e placenta for glucuronidase

FISHMAN: I don't recall the figures which we obtained. They were not excessive for the human placenta.

GOUGH. Could glucuronidase activity in the uterine endometrium

FISHMAN: Very little is understood of the hormonal state immediately after parturition. There must be quite a violent transformation going on, and that is why we are not certain how to interpret the stilbœstrol factor. But this is one situation where œstrogen has an effect. Many people try to explain all of the behaviour of β-glucu-

endometrium of pregnant women?

FISHMAN: As I understand it, there is a decidual tissue which is not pure endometrium any more, because the growth of the placenta pre-

because we haven't been able to construct a satisfactory assay method. However, we have seen some very interesting phenomena in ascitic fluid or pleural fluid taken from patients with cancer. We usually

that there is an inhibitory effect of the fluid upon the cellular glucuronidase.

BUSH: Are there changes of glucuronidase activity with other hormones?

FISHMAN: Progesterone given to castrate female mice has no effect on uterine glucuronidase. Very little is known with respect to nonœstrogenic hormones.

GOUGH: Have you studied glucuronidase in an anovulatory cycle, when there is no pregnanediol and no temperature rise?

FISHMAN: No.

FOLLEY: Is the enzyme present in the urine?

FISHMAN: Yes.

WILLIAMS-ASHMAN: When you administer œstrogens to ovariectomized animals, how long do you have to administer them before you can observe any change in the uterine β-glucuronidase activity?

FISHMAN: That depends upon the mode of administration. The dosage was regulated to correspond exactly with the regular vaginal smear assay technique for œstrogens, so that they were given a divided dosage over three days and killed on the fifth day. But we did an experiment where a single injection of œstrogen dissolved in aqueous alcohol was given. The glucuronidase level was elevated about 20 per cent after one day, but by the third day it was down again.

There was one fact which may have some bearing on the time relationship of œstrogen action and β-glucuronidase response. The Astwood technique for assaying œstrogens involves the measurement of the increment in uterine weight of immature mice receiving *œstrogen*, and that increase in weight is due almost entirely to accumulation of water. Cell division, as I understand it, starts some time later. During this phase when water accumulates, the first six hours, there is no change in glucuronidase activity per gram of tissue, so that the β-glucuronidase response must occur after the phase in which water accumulates, and might be related to the cellular division in this instance.

ENZYMES IN THE CORPORA LUTEA OF THE RAT DURING PREGNANCY AND LACTATION*

R. K. MEYER

THE activity of succinic dehydrogenase, malic dehydrogenase, adenosine triphosphatase, alkaline and acid phosphatases and anaerobic glycolysis enzymes was determined in homogenates of the corpora lutea of pregnant and lactating rats. All enzymes varied in activity in both reproductive phases. Especially noteworthy were the increases in succinic dehydrogenase, malic dehydrogenase and anaerobic glycolysis during the first eleven days of pregnancy. The maximum values for these enzymes were found during the time that the corpora of pregnancy were attaining their maximum weight.

Acid phosphatase and adenosine triphosphatase declined in activity in the corpora of pregnancy during the first eleven and fifteen days, respectively. The greatest activity for the enzymes was observed during the last six days of pregnancy.

Anaerobic glycolysis, acid phosphatase and adenosine triphosphatase exhibited relatively little change in levels of activity during pregnancy, when compared with malic and succinic dehydrogenase. However, the activity of adenosine triphosphatase and alkaline phosphatase in the degenerating corpora of pregnancy was much greater than that found when the corpora were functional. The activity of the other enzymes in the degenerating corpora of pregnancy was decreased markedly soon after parturition.

Histochemical studies showed that alkaline phosphatase in the corpora of both pregnancy and lactation is largely concentrated in the vascular tissue, with relatively little, if any,

*The data presented in this series of reports are the results of the cooperative efforts of my colleague, Dr. W H McShan, our graduate students, and Dr E. G. Shipley

in the lutein tissue. This observation serves to illustrate the importance of histochemical studies for the localization of enzymes in specific cells, and in interpreting data obtained by biochemical determinations of enzyme activities of an organ.

The succinic dehydrogenase activity of the corpora lutea of pregnant rats hypophysectomized on the eighth day of gestation and killed on the twelfth day is decreased when compared with that of corpora of normal controls in the same stage of pregnancy. However, the enzyme activity in the corpora of the rats hypophysectomized on the twelfth day of pregnancy and killed on the fifteenth day, is like that found in control rats.

The data suggest that for the first eight to ten days of gestation in the rat the activity of succinic dehydrogenase of the corpora is dependent on the pituitary gland, and that after the tenth day the placenta is largely, if not wholly, responsible for maintaining the activity.

During lactation the succinic dehydrogenase activity of the corpora decreases markedly after hypophysectomy on the fourth, eighth or twelfth days, which demonstrates the importance of the pituitary gland in the maintenance of the function of this enzyme during lactation.

Definite patterns of changes in enzyme activity occur in the corpora lutea of the rat under the natural and experimental conditions studied. These patterns are correlated with anatomical and functional changes in the gland, and with the level of trophic hormones acting on the gland.

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of involution which follows weaning there is a fall in the enzyme concentration. I wonder if the enzyme content of both these tissues is under

views

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concern

synthe

MEYER: It is certainly true that the correlations are there. With regard to the action of luteotrophin, during lactation when we have this large increase in alkaline phosphatase, larger than during pregnancy, the corpora are small. At the same time the mammary glands are, of course, very active, so that makes a good correlation. It may be that during lactation the output of luteotrophin from the pituitary

during pregnancy and the absence during lactation. The succinic dehydrogenase and alkaline phosphatase are high, for example, in these small corpora lutea during lactation. In pregnancy the corpora will increase 400 per cent in size. We have not been able to explain what the "survival value" is of this large corpus luteum. It may be that during the latter half of pregnancy in the rat the corpus luteum is acting as a supplementary producer of adrenal cortical hormones, because one can at least build up a case that the metabolic stress during the latter half of pregnancy is tremendous and the corpus luteum may not be producing much progesterone at that time. This is indicated by the studies of Atkinson and Hooker on the mouse. As we know, progesterone will maintain pregnancy in the adrenalectomized animal, and the corpora lutea are sufficient during pregnancy to maintain life in the adrenalectomized rat, so there is undoubtedly an interplay between these two tissues.

pregnancy.

MEYER: Everett's work shows the importance of studying cholesterol content during these functional phases, and that deserves further study.

KOCHAKIAN: There is a general trend towards the view that alkaline phosphatase is involved in reorganization ("reshuffling"), breakdown and build-up of proteins rather than the splitting of phosphate. I think your thoughts fit right in with this concept

FISHMAN It is certainly easier to imagine that these changes in enzyme concentration are associated with the synthesis of cell material rather than with their breakdown, however we may measure the activities *in vitro*. I was wondering what might happen to an

ENZYMES IN THE PLACENTOMA OF THE RAT

R. K. MEYER

Placentomata produced artificially in the pseudopregnant rat were examined for the presence and quantities of alkaline and acid phosphatases, β-glucuronidase, succinic and malic dehydrogenases, and nucleic acids. Tissue was collected for analyses on the sixth, seventh, ninth, tenth, eleventh and twelfth days after the uterus was traumatized.

Alkaline phosphatase activity increases rapidly up to the seventh day after the uterus has been traumatized. At this time the formative stage of the placentomata has been reached. During the involutional and degenerative phases (ninth to twelfth days) the alkaline phosphatase declines to very low levels. Acid phosphatase, however, is low when the placentomata are developing, reaching high values at the time necrosis is most marked.

Both nucleic acids are greatest in concentration on the seventh day, and decline slowly through day eleven.

Succinic and malic dehydrogenase and β-glucuronidase are found most active during involution and necrosis.

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DISCUSSION

... determinations on alkaline phosphatase in mammary

Tickner Can you tell me at what stage of pregnancy the adrenals were removed?

Meyer· The adrenals were removed on the 12th day.

Bush. How did you measure the progesterone?

Meyer The data are from Laqueur and Koets They didn't measure progesterone directly. They computed it from the ketosteroids.

Bush Right in the corpora lutea?

Meyer: Yes. They also determined the total lipid and cholesterol content and water content in the corpora of pregnancy.

Folley: Have you studied the adrenal cortex?

Meyer. No, we haven't.

Folley: We have studied the alkaline phosphatase in the whole kidney—we've not attempted to differentiate between the cortex and medulla—and we find that the alkaline phosphatase level in the kidney is surprisingly constant in pregnancy and lactation.

Kochakian: I would expect as much from œstrogen studies.

Folley· Is your alkaline phosphatase fully activated with magnesium? Have you worked out the percentage activation that can be brought about by magnesium to see whether there are any differences there?

Meyer. I think that we have worked out the optimum conditions, though I can't remember the exact data. This was done three years ago.

Kochakian Has anybody actually shown that you can get a difference using full activation?

Folley I don't think anyone has shown that, but differences in the percentage activation of the homogenates have been observed, though these were not sufficiently great so that different trends were observed at full activation from those shown by the enzyme in its natural state.

Kochakian: We have some phosphatase studies, with and without magnesium, and no divergence in results has been observed as yet.

greater activity than has been demonstrated. Furthermore, in these homogenates we have to remember that we have disrupted the cytoarchitecture of the cell and that the enzymes may not be working at the potential activity which they would have in the intact cell.

adrenalectomized pregnant rat. Will the animal's corpus luteum protect it?

MEYER: We have been doing some experiments along that line. If you adrenalectomize pregnant rats the pregnancy will be maintained, although parturition is difficult. The corpora lutea do not undergo

parent animal,

ophy to 7 and 8

This indicates

as an end organ.

FOLLEY: Have you done any experiments on making rats pseudopregnant and then giving lactogenic hormone? Might it put up the alkaline phosphatase, for instance, above the value you get in late pregnancy?

on it.

those.

MEYER: Yes. We made similar studies, but not as completely, with succinic dehydrogenase and adenosinetriphosphatase during pseudopregnancy in rabbits. We saw much the same picture.

possibilities.

MEYER: We haven't been doing anything with lutein tissue. It's on the programme, but we were, so to speak, cutting our teeth on liver.

THE EFFECTS ON ENZYMES OF ANDROGENS AND GROWTH HORMONE*

CHARLES D. KOCHAKIAN

Dr. Kochakian presented data from many experiments by himself and co-workers. Most of this material has been published elsewhere, and summaries of these publications, prepared by Dr. Kochakian, are given below.

THE EFFECT OF CASTRATION AND TESTOSTERONE PROPIONATE ON D-AMINO ACID OXIDASE ACTIVITY IN THE MOUSE. *Science* 98, 89 (1943). L. C. Clark, Jr., C. D. Kochakian and R. Phyllis Fox.

THE mouse kidney loses part of its ability to oxidatively deaminate D-alanine as a result of castration. The administration of testosterone propionate not only restores this property, but increases it above normal.

THE EFFECT OF CASTRATION AND TESTOSTERONE PROPIONATE ON THE "ALKALINE" AND "ACID" PHOSPHATASES OF THE KIDNEY, LIVER, AND INTESTINES OF THE MOUSE. *J. biol. Chem.*, 153, 669-674 (1944) Charles D. Kochakian and R. Phyllis Fox

There was a decrease in the "alkaline" (pH 9 8) phosphatase accompanied by an increase in the "acid" phosphatase (pH 4·9) in the kidneys of normal and castrated mice treated for 35 and 115 days with a subcutaneous pellet of testosterone propionate. Castration resulted in a decrease in both of the enzymes in about the same proportion as the diminution in kidney weight.

The enzymes of the liver and intestine were not significantly changed as a result of testosterone propionate treatment or castration.

*The work reported in th . . . . [illegible]

maintain pregnancy depend in any way on the size of the litter? Supposing you happened to have a small litter or took some away, would one corpus luteum be enough?

MEYER: I don't know. You mean that if you do a unilateral hysterectomy during pregnancy, the number of corpora lutea needed would be less than one? That we haven't tried.

BUSH: How does the activity of ADT-ase compare with that of muscle?

MEYER: It is low.

3β: 17α-diol caused decreases of 33 to 39 per cent. The changes were not related to changes in kidney weight or amount of material absorbed.

The increases in arginase activity represented greater amounts of enzyme and not a production of arginase activators.

The kidneys of the castrated mice contained the same amount of total arginase but greater amounts per gram of tissue than did those of the normal mice.

THE EFFECT OF DOSE AND NUTRITIVE STATE ON KIDNEY ARGINASE AFTER STEROID STIMULATION. *J. biol. Chem.*, 161, 115-125 (1945)
Charles D. Kochakian

The increase in arginase activity obtained in the kidneys of castrated mice treated for 30 days with various steroids implanted subcutaneously as pellets is related to the amount and the chemical structure of the compound absorbed. There is at first a decrease in arginase activity of the kidney which occurs during the phase when the kidney is increasing in size to its maximum response. This initial phase is followed by a rapid increase in arginase activity. These two phases are altered by the chemical structure of the steroid. Testosterone, testosterone propionate, androstane-3α:17α-diol, and androstan-17α-ol-3-one produce almost identical responses per mol of steroid absorbed. The introduction of the 17-methyl group (17-methyltestosterone and 17-methyl-androstane-3α· 17α-diol) causes a rapid initial increase instead of a decrease in arginase activity, which then continues at a slower rate of increase until it becomes identical with that of the former compounds.

Undernutrition does not affect the ability of the steroids to stimulate arginase activity per gram of tissue, but decreases the total increase because of the smaller increase in kidney size.

It is suggested that the increased arginase activity is related to synthetic processes such as protein anabolism and glycocyamine formation.

The tissues of the older mice contained more "alkaline" phosphatase than those of the younger animals.

HISTOCHEMICAL STUDY OF "ALKALINE" PHOSPATASE OF THE KIDNEY OF THE CASTRATED MOUSE AFTER STIMULATION WITH VARIOUS ANDROGENS. *Amer. J. Physiol*, 152, 257-262 (1948) Charles D Kochakian.

The distribution of the "alkaline" phosphatase of the kidney of the mouse has been studied after treatment with eighteen different steroids, many of which were studied at several dose levels. There was a progressive decrease in the enzyme from the distal end of the proximal convoluted tubule towards the glomerulus, with a slight increase in concentration at the glomerular end. These changes paralleled the increase in kidney size under steroid stimulation, so that at maximum stimulation the kidney showed varying degrees of depletion of the enzyme in its nephrons.

THE EFFECT OF CASTRATION AND VARIOUS STEROIDS ON THE ARGINASE ACTIVITY OF THE TISSUES OF THE MOUSE. *J. biol. Chem*, 155, 579-589 (1944) Charles D Kochakian.

Mice weighing 16 5 to 19·5 g. were castrated, and one month later 14$\pm$1 mg. pellets of various steroids were implanted subcutaneously. Arginase determinations were made 10 and 30 days later. None of the steroids affected the enzyme content of the liver or intestine, but many of these compounds markedly increased and a few decreased the arginase content of the kidneys. The order of change in per cent difference per gram of kidney tissue for the 30 day experiments was as follows: methyltestosterone, 632; testosterone, 584; testosterone propionate, 308; 17-methyl-androstane-3α:17α-diol, 269; androstan-17α-ol-3-one, 135; α-œstradiol, 88; androstane-3α:17α-diol, 71; 17-vinyltestosterone, 55; testosterone-3-acetate-17-propionate, 35. Much greater changes were obtained when the values were calculated on the basis of total tissue. Eighteen other compounds had no effect, and *iso*androsterone, 17-methylandrostene-3β· 17α-diol, and 17-methylandrostane-

3β: 17α-diol caused decreases of 33 to 39 per cent. The changes were not related to changes in kidney weight or amount of material absorbed.

The increases in arginase activity represented greater amounts of enzyme and not a production of arginase activators.

The kidneys of the castrated mice contained the same amount of total arginase but greater amounts per gram of tissue than did those of the normal mice.

THE EFFECT OF DOSE AND NUTRITIVE STATE ON KIDNEY ARGINASE AFTER STEROID STIMULATION. *J. biol Chem*, **161**, 115-125 (1945) Charles D. Kochakian.

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Undernutrition does not affect the ability of the steroids to stimulate arginase activity per gram of tissue, but decreases the total increase because of the smaller increase in kidney size.

It is suggested that the increased arginase activity is related to synthetic processes such as protein anabolism and glycocyamine formation.

EFFECT OF TESTOSTERONE PROPIONATE AND GROWTH HORMONE ON THE ARGINASE AND PHOSPHATASES OF THE ORGANS OF THE MOUSE. *Amer. J. Physiol.*, 155, 262-264 (1948) Charles D. Kochakian and Constance E. Stettner.

Mice were castrated at 17- to 19-grams body weight and one month later were (1) implanted subcutaneously with a 14–15 mg. pellet of testosterone propionate, (2) injected subcutaneously with 1·1 rat U/day of growth hormone, and (3) treated simultaneously with both hormones for 10-, 20- and 34-day periods. Testosterone propionate produced the expected marked increase in kidney arginase, small increase in "acid" (*p*H 5·4) phosphatase and marked decrease in "alkaline" (*p*H 9·8) phosphatase activities. Growth hormone was ineffective, but when administered simultaneously with testosterone propionate, it decreased the arginase-stimulating effect of the androgen to one-half.

The liver enzymes were not affected by either of the hormones. The small increases in liver size were accompanied by proportionate increases in the enzyme activities.

THE EFFECT OF ANDROGENS AND HYPOPHYSECTOMY ON ARGINASE AND PHOSPHATASES OF THE KIDNEY AND LIVER OF THE RAT. *Arch. Biochem.*, 29, 114-123 (1950) Charles D. Kochakian and Evangeline Robertson.

Androgens produced a small increase in weight and an increase in proportion to dose in arginase activity of the kidney of the castrated rat. The "alkaline" phosphatase was slightly increased and the "acid" phosphatase changed in proportion to the weight. The arginase activity of the liver was not affected by the androgens, but the alkaline phosphatase showed an irregular but persistent small increase.

Hypophysectomy of adult male rats resulted in a marked decrease in arginase, a slight decrease in acid phosphatase, and an increase in alkaline phosphatase activity of the liver. All of these enzymes of the kidney rapidly decreased. Testosterone did not affect the liver enzymes but restored the arginase and alkaline phosphatase activities of the kidney.

EFFECT OF CASTRATION AND ANDROGENS ON BODY AND ORGAN WEIGHTS, AND THE ARGINASE AND PHOSPHATASES OF KIDNEY AND LIVER OF THE MALE SYRIAN HAMSTER. *Amer. J. Physiol.*, 153, 210-214 (1948) Charles D. Kochakian, Mary N. Bartlett and José Gongora.

Castration caused a decrease in the size of the seminal vesicles and prostates but no change in the kidney or liver. The administration of testosterone propionate by injection or by the subcutaneous implantation of a pellet for 20 and 140 days increased the seminal vesicles and prostates of castrated hamsters to greater than normal but did not affect the size of the kidney or liver. Pellets of testosterone and 17-methyltestosterone implanted subcutaneously for 20 days produced similar responses.

The arginase of the kidney increased as a result of castration and decreased to normal with the various androgen treatments. The "alkaline" phosphatase, on the other hand, decreased after castration and was restored to normal with androgen treatment. The "acid" phosphatase of the kidney and the arginase and phosphatases of the liver were not affected by castration or by the androgen treatment.

EFFECT OF CASTRATION AND STEROIDS ON THE ARGINASE AND PHOSPHATASES OF THE ORGANS OF THE GUINEA PIG *Amer. J. Physiol*, 155, 251-254 (1948) Jane Harrison Humm, Charles D Kochakian and Mary N. Bartlett.

Male guineapigs were castrated at about 250 g. body weight. Thirty-five days later they were implanted subcutaneously with pellets of the following steroids: 17-methyltestosterone; testosterone; testosterone propionate; 17-methylandrostan-17α-ol-3-one; androstan-17α-ol-3-one; 17-methylandrostane-3α:17α-diol; and androstane-3α·17α-diol. The dose of steroid was varied by the number of pellets implanted. Castration produced a decrease in the arginase activities of the kidney after 60 days, but not after 120 days. None of the steroids produced any remarkable changes. The greatest increase, 79 per cent, was produced by 17-methyltestosterone, while testosterone was completely ineffective. The administration of a relatively large dose, 12.5 mg./day, of testos-

terone propionate for 14 days produced only a 38 per cent increase. Castration produced a decrease in the "alkaline" phosphatase of the kidney, which was restored toward normal by the various steroids. None of the enzymes of the liver or the "acid" phosphatase of the kidney were affected by castration or the steroids.

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DISCUSSION

FOLLEY: I was very interested in your theory of the possible function of arginase in the kidney. That is something of a mystery, of course. Have you done any experiments designed to get actual evidence for the transfer of the amino group to glycocyamine?

KOCHAKIAN: We planned it, but other things have been more pressing.

FOLLEY:

KOCHAKI

attracted b

YOUNG.

You compared, for instance, hypophysectomized animals and normal animals.

KOCHAKIAN. The nitrogen metabolism was determined on those animals. In the hypophysectomized animals food intake will go down, while the castrated animals will maintain body weight and nitrogen

The hypophysec-

g. per day over a

veight and nitrogen

equilibrium.

YOUNG: Your first experiment was made on groups of animals which were not consuming the same amounts of food?

KOCHAKIAN: That's right.

Young: Do you find no change in blood amino-acid with growth hormone?

Kochakian: I haven't done it with growth hormone; just with testosterone.

Folley: Is there any effect of androgens *in vitro* on arginase and phosphatase?

Kochakian: We did add testosterone to homogenate and got nothing Also we did some experiments with adrenal cortex extract and got nothing.

Greenbaum: In your experiments with androgen you got a very

questioning whether it has anything to do with urea formation in the liver.

Storey: I think it is a very interesting suggestion, and it does raise the possibility that some of these enzymes may have a function other than the rather obvious one that we have always concluded that they have. I was thinking too of the suggestion that was thrown out yesterday about glucuronidase

Kochakian: The work that I am going to talk about this afternoon on the adrenal cortex suggests that arginase hasn't anything to do with urea formation· it is probably more concerned with internal reshuffling of protein.

Folley. What do you think of Edlbacher's theory of the function of arginase?

Kochakian: That was the hypothesis which first gave me some support in these views. I gather, however, that Fraenkel-Conrat thinks that this is a specialized type of synthesis.

Folley: We've done a lot of studies on the mammary gland, some of which Dr. Greenbaum will mention this afternoon, and we are faced with the same problem there—finding a function for the mammary gland arginase Dr Greenbaum rather clings to the classical idea that the mammary arginase is concerned in the formation of urea. However, there are difficulties to be faced, since in preliminary experiments he hasn't been able to observe urea formation in mammary gland slices in the rat. Another difficulty is that in herbivores, particularly ruminants, the mammary gland arginase level is very low. Arginase doesn't seem to play a very important part in lactation in herbivores We find the same species differences in the liver arginase levels. Rat and mouse liver have a much higher content of arginase than the liver of the rabbit, goat, or cow. Have you any observations on this?

Kochakian: As I remember, our guinea pigs showed low arginase activity.

FOLLEY: We haven't got [illegible]

meat diet slightly lowered the liver arginase levels.

KOCHAKIAN: How long did you feed it?

FOLLEY: Three weeks.

KOCHAKIAN: [illegible]
a source of prot[illegible]
on arginase. If [illegible]
you get a possible slight increase, but after about three weeks you do get a definite increase. Lightbody also found that same effect several years ago.

FOLLEY: Dr. Greenbaum, you ran some experiments with an ordinary high protein diet, didn't you?

GREENBAUM: Yes. Our results didn't really confirm Dr. Lightbody's finding. We did get an increased [illegible] 7 days on a [illegible] another 10 [illegible] hat an animal which has been chewing up protein at a high level for so long is going to have a very increased arginase.

KOCHAKIAN: That's right. And that's what makes me even more suspicious of the urea function for arginase.

COMPARISON OF β-GLUCURONIDASE ACTIVITY IN TISSUE OF FŒTAL, NEW-BORN, AND INFANT ANIMALS WITH THOSE OF THE MOTHER (MOUSE, DOG, AND HUMAN)

W. H. FISHMAN

It has been reported by Dr. Levvy and his co-workers in Edinburgh* that the activity of β-glucuronidase in a tissue was correlated with the state of cellular proliferation and growth. Thus, for example, the tissues of fœtal, new-born, and infant mice exhibited high glucuronidase activity when compared to the corresponding tissues of the mother. These investigators employed phenyl glucuronide as substrate and subjected the homogenate to incubation at an acid *p*H for thirty minutes, followed by an ammonium sulphate precipitation procedure, before enzyme assay.

In repeating these experiments, we prepared fresh tissue homogenates which were centrifuged at high speed and the supernatants were assayed for β-glucuronidase activity, employing phenolphthalein β-glucuronide as substrate.

The enzyme activity of the kidney, spleen, brain, stomach, and heart of fœtal mice and one-, two-, and three-week-old litter-mates, never showed an increased activity as compared with the organs of the maternal organism. Similar results were found in the dog, comparing fœtal and maternal tissue. Human embryonic tissue exhibited less glucuronidase activity than had been found in organs of adult humans. It would appear that our findings are at variance with those of Dr. Levvy, and we have suggested to him that the discrepancy may lie either in the different experimental techniques which our two laboratories employ or in strain differences.

*Levvy, G. A., Kerr, L. M. H., and Campbell, J. G. (1948) *Biochem. J.* 42, 462

DISCUSSION

MILLS: Our experience has been not unlike that of Dr. Fishman. The levels of liver glucuronidase activity in embryonic or young rats (birth to 3 weeks) are less than in adults. We have used an extraction procedure similar to that of Levvy, using phenyl and phenolphthalein glucuronide for assay, and also a technique like that of Fishman, and the results are very similar. In the case of mice, we have obtained results very similar to those of Levvy—namely a higher liver glucuronidase activity in young mice than in adult, and we feel that the difference between rats and mice is a species difference.

FISHMAN: As I said yesterday, I feel that on the basis of the glucuronidase concentration to uterine nitrogen relationship, that the amount of protoplasm *per se* is not the important factor, and I think Dr. Mills is chiefly of that opinion. It would look more as if there is some more [illegible]

to growth changes alone.

RELATION OF GLUCURONIDASE TO ACTION OF GONADAL HORMONES

R. K. MEYER

THE β-glucuronidase activity of the livers of mice, rats and frogs receiving α-œstradiol or diethylstilbœstrol is not significantly higher than that of livers of control animals.

Doses of œstrogen which cause pituitary hypertrophy in ovariectomized mice and hypertrophy of the seminal vesicles in castrated rats did not elevate the glucuronidase activity in these tissues.

When graded doses of diethylstilbœstrol are given to ovariectomized mice there is a proportional increase in the activity of glucuronidase and the growth of the uterus at the several dose levels used.

Doses of α-œstradiol administered to ovariectomized rats in amounts sufficient to maintain the uterus at near normal weight were insufficient to maintain the glucuronidase activity above the castrate level.

Small doses of progesterone inhibit the stimulating effect of diethylstilbœstrol on weight and glucuronidase activity of the uterus ; large doses cause stimulation of uterine weight and glucuronidase, particularly when accompanied by minute quantities of œstrogen.

Progesterone inhibits the action of glucuronidase *in vitro.* This effect is not shown with the other steroids studied, i.e., [illegible] ·one, 1-methyl-α-œstra- [illegible] to the possible physiological factors associated with elevated glucuronidase activity. It was suggested that glucuronidase may be involved in the accumulation of water in tissues through its possible role in the metabolism of intercellular ground substances.

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STAFFORD, R. O. (1949). *The Influence of Hormones on Enzymes in Some Endocrine Target Tissues* Thesis submitted for the degree of Doctor of Philosophy, Univ. Wisconsin.

DISCUSSION

FISHMAN: I'm very much interested in the water hypothesis because it may help explain some of the results that we've found in toxæmic women. To the gynæcologist the first symptom that presents itself in

nancies and abnormal pregnancies he seemed to find serum glucuronidase correlated with this gain in weight in several women. That observation made us look into the current theories of causation of toxæmia in pregnancy. As a rule the reports show that the urine of these women contains abnormally low amounts of pregnanediol and other steroids. The hypothesis which is widely accepted is that there is somehow a breakdown of the normal production of steroids. I think those data can be explained equally well on the basis that the steroids are formed in the normal amount but are not being excreted. They are being retained in the tissues of these women, with a resulting increase in water. And when we come to examine the literature as to what relationship there may be between œstrogens and water retention in the tissues, we find that certain people in this country, for example, have shown a very good relationship between œstrogen and water retention in tissues The sexual skin of the monkey becomes turgid during œstrus and it has been shown that there may be increased amounts of hyaluronic acid formed. All this has made us suspect a relationship between œstrogen, glucuronidase and mucin. So in the experiments that you described today it seems to me that there is direct evidence in support of these relationships. The fact that you find that 0 5 μg. of œstrogen is necessary for maintaining the blood level makes me think that perhaps in the early increase in uterine weight in the Astwood procedure, there is not sufficient œstrogen to produce an appreciable glucuronidase response. Certainly the amounts that we use under those assay conditions are well below 0·1 μg, about 0·05 μg.

FOLLEY: Did you try deoxycorticosterone glucoside in the *in vitro* experiments? That is a water-soluble derivative, and would do away

FOLLEY: Is that a detergent?

MEYER: Yes.

BUSH. I think that you get quite stable emulsions, at any rate with progesterone, using very minute quantities of alcohol. You can dissolve it in 80 per cent alcohol and then add a considerable volume of water so that the percentage of alcohol finally is only around 2 per cent, and then you get an emulsion which is stable and can be used for injection.

MEYER. We did some preliminary experiments on suspensions from a quantitative point of view. We didn't know how much we had in solution, so we settled on 10 per cent alcohol because these concentrations gave a water clear solution. Some investigators have just ground up the steroids and homogenized the tissue and then recorded the effects, on the assumption that the maximum was in solution.

WILLIAMS-ASHMAN: Have you tried ethylene glycol mono-ethyl ether as a solvent? It is supposed to be non-toxic.

MEYER: No.

FOLLEY: Has anyone tried to find a histochemical method for glucuronidase?

MEYER: That is what Friedenwald and Becker used.

FOLLEY: What is the method?

FISHMAN: They used 8-hydroxyquinoline glucuronide as the substrate and incubated slices of fresh tissue in the presence of this substrate plus an excess of ferrous iron. The iron reacts with the liberated hydroxyquinoline, precipitating in the tissue. Ultimately this iron is converted to Prussian blue.

Other people in Boston are developing histochemical methods for glucuronidase, and they have told me that glucuronidase stains well in epithelial, endometrial and glandular tissue, as well as in the epithelial malignant cell.

THE GROWTH INHIBITING ACTION OF CANCER PRODUCING SUBSTANCES IN RELATION TO HORMONAL CONTROL OF PROTEIN AND CARBOHYDRATE METABOLISM

L. A. ELSON

THE body growth and tumour growth inhibiting action of cancer producing substances such as 1:2:5:6-dibenzanthracene, 4-dimethylaminostilbene, etc., has been shown to be related to the protein content of the diet (Elson and Warren, 1947; Elson, 1948). In rats maintained on a high (20 per cent) protein diet, injection of the carcinogen usually has little immediate effect on body growth, although a delayed action resulting in rapid loss of weight, often followed by death, may occur later. Animals maintained on a low (10 per cent) protein diet, however, usually respond to the injection by an immediate, often prolonged, retardation of growth. The mechanism of the growth inhibiting action of these substances appears to be different from that of œstrogenic hormones like stilbœstrol, which was not found to be influenced by the protein content of the diet (Elson and Warren, 1947) and, unlike that of carcinogens, may be caused by antagonism of the anterior pituitary growth hormone (see Griffiths and Young, 1942).

It is suggested that the growth inhibition caused by cancer producing substances is a result of interference with protein metabolism, possibly directly with some enzyme process concerned in protein synthesis or through an action on nucleic acids which may act as intermediaries in the synthesis of special proteins, since Elson and Harris (1947) have shown an interference with the normal ratio of pentosenucleic- to deoxypentosenucleic acid in the livers of rats treated with 1:2:5:6-dibenzanthracene.

Another effect of this carcinogen on rat liver is to cause an crease in ascorbic acid content. This increase was found be greatest in animals maintained on a 10 per cent protein et, with which the maximum growth-inhibitory action of the mpound was observed (Elson, Kennaway and Tipler, 1949).

Thus, as a result of this and other evidence concerning the xicity of 1:2:5:6-dibenzanthracene in rats maintained on gh and low protein diets (Elson, 1949), it appears possible at the carcinogens may interfere with the normal hormonal gulation of protein and carbohydrate metabolism, not, as likely in the case of œstrogens, by antagonism of protein abolic hormones themselves, but by a more direct interrence with the action of some protein synthesizing enzymic stems under their control. It is thus possible that the rcinogens may be preventing the utilization of energy rich osphate bonds for protein synthesis, and the energy rich osphate is then available for an increased carbohydrate etabolism, of which there is some evidence in treated imals. Indeed, the increased liver ascorbic acid may well related to this increased carbohydrate metabolism.

There is a considerable amount of evidence that carcinogens ch as the polycyclic hydrocarbons and aminostilbene deriatives exert their biological action through a "toxic" etabolic product, and the action of these carcinogens can us be considered as a dynamic process in which the rate of rmation of the toxic metabolite depends to some extent on e rate of metabolism of the animal as a whole. The toxic etabolite itself upsets the balance of energy distribution etween protein and carbohydrate metabolism which is ormally regulated by hormonal influence.

The possibility that glucuronic acid and glucuronidase play n important part in these metabolic processes is suggested y the observations of Elson, Goulden and Warren (1946). n studying the excretion of aromatic amines it was found that e simpler amines such as aniline, 4-chloroaniline, etc., when dministered to rats in small doses are excreted almost entirely s ethereal sulphates of the corresponding phenol derivatives.

These amines are not carcinogenic. Compounds containing more than one aromatic ring, however, such as 4-amino-diphenyl, benzidine and 4-aminostilbene, were found to be excreted as glucuronides and some of the free phenol derivatives were also found in the urine. Benzidine, 4-amino-stilbene and the *N*-dimethyl-4-amino-diphenyl have been shown to be carcinogenic; aminostilbene and its derivatives in particular producing a large variety of tumours in many organs of the rat.

In this connection I would like to suggest that glucuronic *acid formation and the role of glucuronidase in animal* metabolism may not be exclusively concerned with the so called detoxification mechanisms, but may also be that of providing a transfer mechanism for conveyance of a fat-soluble but water-insoluble substance such as a steroid hormone from one organ in the body to others on which it is required to act. The substance is transferred by being converted into a water-soluble glucuronide, which may no longer show hormone activity, and this is carried in the blood stream, and, in this water-soluble form, is able to enter the cells of various organs. The active hormone is then liberated *in situ* in that organ by means of the glucuronidase present in the cell. On this conception the rather vague relation of *glucuronidase to growth may be explained by the growth* effects being really caused by the liberated hormone, and thus only indirectly related to the enzyme. Thus we would have, instead of the more usually accepted process of a hormone regulating enzyme activity, in this case, the hormone actively regulated by the enzyme. This regulation could be controlled by the varying amounts of glucuronidase in the different tissues and/or by the presence or absence of an inhibitor of glucuronidase. The presence of such inhibitors in blood serum, etc., *is probably significant in this respect.* An

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the main form of excretion as ethereal sulphates to that of glucuronides occurs, may be highly significant, since transference as the water-soluble glucuronide and liberation of active carcinogen by the glucuronidase present in the various tissues could then occur. Excretion as glucuronide may thus be of marked importance for the production of tumours in such a number of diverse organs as occurs with this type of carcinogen.

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DISCUSSION

YOUNG: Was the food intake observed in all these experiments?
ELSON: It was observed in some of the later experiments; in the earlier

inhibition, but not all, because some animals maintain or even increase their food intake under treatment, when their growth is completely inhibited.

YOUNG: Did you analyse the total body content of protein or of fat?

ELSON: No. We didn't.

YOUNG: The overall weight of the animal may not necessarily be significant with respect to protein metabolism.

FOLLEY: Are these carcinogenic compounds œstrogenic at all in large doses?

ELSON: No. There is a slight suggestion, but very slight.

FOLLEY: Do you know of any effect of these compounds upon pituitary cytology?

ELSON: No

YOUNG: You think that stilbœstrol suppresses the activity of the [illegible]

These amines are not carcinogenic. Compounds contain more than one aromatic ring, however, such as 4-ami diphenyl, benzidine and 4-aminostilbene, were found to excreted as glucuronides and some of the free phenol d vatives were also found in the urine. Benzidine, 4-ami stilbene and the *N*-dimethyl-4-amino-diphenyl have b shown to be carcinogenic; aminostilbene and its derivat in particular producing a large variety of tumours in ma organs of the rat.

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The observation that, in passing from the simpler non carcinogenic amines to the carcinogenic ones, a change fro

THE EFFECTS ON ENZYMES OF ADRENAL CORTEX, DIET, ŒSTROGENS, AND EXPERIMENTAL DIABETES*

CHARLES D. KOCHAKIAN

Dr. Kochakian presented data from many experiments by himself and co-workers. Most of this material has been published elsewhere, and summaries of these publications, prepared by Dr. Kochakian, are given below.

Adrenal Cortex

THE EFFECT OF ADRENALECTOMY, ADRENAL CORTICAL HORMONES, AND TESTOSTERONE PROPIONATE PLUS ADRENAL CORTICAL EXTRACT ON THE ARGINASE ACTIVITY OF THE LIVER AND KIDNEY OF THE RAT. *J. biol. Chem.*, 169, 1-6 (1947) Charles D. Kochakian and Virginia N. Vail.

ADRENALECTOMY decreased the fasting urinary nitrogen excretion and the arginase activity of the liver and kidney of young (150 g.) adult male rats. The administration of 1 per cent sodium chloride as drinking water was ineffective, and deoxycorticosterone acetate, 1 mg. per day, had a slight alleviating effect on the decrease in kidney arginase only. The administration of adrenal cortical extract (aqueous, Upjohn) at hourly intervals for 8 hours on the 5th postoperative day greatly increased the urinary nitrogen but did not affect the liver arginase, and partly restored the kidney arginase. Previous treatment with testosterone propionate, 2·5 mg. twice per day, did not alter the effects of the adrenal cortical extract but greatly increased the kidney arginase.

*The work reported in this paper was support[illegible]

pituitary extract could cause resumption of growth in animals where the growth had been depressed or prevented by administration of œstrogens.

KOCHAKIAN: *I have often thought it was more an effect upon the food intake.*

FOLLEY: But you do get cytological changes in the pituitary on œstrogen administration. One would think there would be a profound *effect on the secretory mechanism*

YOUNG: I am sure there are profound effects on the pituitary-secretory mechanism with respect to gonadotrophin, for instance, but I don't think as regards growth hormone that there is any clear evidence. We have a good deal of unpublished work which suggests that there may be some slight effect which is not merely the result of changes in the food intake, but the differences are so small as to be very doubtful

ELSON: What do you feel about the possibility of the inhibitory action being through an effect on bone growth?

YOUNG: Griffiths and Young found that growth of bone was depressed but not prevented. It is slow and may go on even when the body weight is falling.

A pellet of 11-deoxycorticosterone acetate produced a small increase in kidney weight and a small decrease in liver arginase, but 11-deoxy-17-hydroxycorticosterone was ineffective.

THE EFFECT OF ADRENALECTOMY, ADRENAL CORTICAL HORMONES, AND TESTOSTERONE PROPIONATE PLUS ADRENAL CORTICAL EXTRACT ON THE "ALKALINE" AND "ACID" PHOSPHATASES OF THE LIVER AND KIDNEY OF THE RAT. *Amer. J. Physiol.*, **150**, 580-587 (1947) Virginia N. Vail and Charles D. Kochakian.

Adrenalectomy slightly increased the "alkaline" (pH 9·8) phosphatase of the liver of young (150 gram) adult male rats. The hourly injection for eight hours of adrenal cortical extract (aqueous Upjohn) on the fifth post-operative day greatly increased this enzyme. The increase occurred at a much faster rate than the increase in glycogen. Histochemical studies demonstrated greater amounts of the enzyme to be present (produced?) in the cytoplasm, walls and nuclei of the liver cells. Previous treatment with testosterone propionate did not alter the effect of the adrenal cortical extract on either the amount of glycogen or the increase in the enzyme activity. Deoxycorticosterone acetate was ineffective.

Adrenalectomy resulted on the fifth post-operative day in a small decrease in the "alkaline" phosphatase of the kidney which was prevented by the administration of 1 per cent sodium chloride as drinking water or by the daily injection of 1 mg. of deoxycorticosterone acetate. The hourly administration for 8 hours of adrenal cortical extract on the fifth post-operative day was ineffective Testosterone propionate, $2 \times 2·5$ mg./day, produced a marked increase in this enzyme.

None of the above treatments produced a significant change in the "acid" phosphatase (pH 5 4) of either the liver or the kidney.

EFFECT OF HIGH PROTEIN AND HIGH CARBOHYDRATE DIETS ON THE ARGINASE AND PHOSPATASES OF THE LIVER AND KIDNEY OF THE NORMAL AND ADRENALECTOMIZED RAT. *Amer. J. Physiol.*, **154**, 489-494 (1948) Charles D Kochakian, Mary N. Bartlett and Jean Moe.

The feeding of either a high carbohydrate (89 per cent)—no protein, a high protein (casein 80 per cent, yeast 10 per cent)

THE EFFECT OF CRYSTALLINE ADRENAL CORTICAL STEROIDS, DL-THYROXINE, AND EPINEPHRINE ON THE ALKALINE AND ACID PHOSPHATASES AND ARGINASE OF THE LIVER AND KIDNEY OF THE NORMAL ADULT RAT. *J. biol Chem.*, 176, 243-247 (1948) Charles D. Kochakian and Mary N. Bartlett.

Aqueous (beef) adrenal cortical extract, lipoextract (hog adrenals), and 11-dehydrocorticosterone acetate produced very marked increases in the "alkaline" (*p*H 9·8) phosphatase of the liver of fasted rats when injected eight times at hourly intervals. The increase in enzyme activity did not parallel the degree of glyconeogenesis. Thyroxine produced a marked depletion of liver glycogen and a decrease in the enzyme. Epinephrine produced a tremendous deposition of liver glycogen but did not affect the activity of the enzyme.

In none of the above treatments were the activities of the arginase and "acid" (*p*H 5·4) phosphatase of the liver or the enzymes of the kidney altered.

CORTICOIDS AND BODY AND ORGAN WEIGHTS, NITROGEN BALANCE AND ENZYMES *J. biol. Chem.*, 190, 481, (1950) Charles D Kochakian and Evangeline Robertson.

The stimulation of rapid glyconeogenesis in mice by corticoids did not alter the arginase activities of the liver or kidneys. On the other hand, a subcutaneously implanted pellet of 11-dehydro-17-hydroxycorticosterone acetate produced significant increases in both tissues after two days. The same increases were obtained when the enzyme was determined with $CoCl_2$ as the enzyme activator or by pre-activation at 50°C with $MnCl_2$. The alkaline phosphatase of the liver was not increased until after 7 days. The body weight of the treated mice decreased very sharply, accompanied by an increased nitrogen excretion, a complete disappearance of the thymus, and a maximal decrease in the size of the spleen; but the food intake was increased about 20 per cent, so that after 7 days the extra protein catabolism was no longer evident and the loss in body weight abruptly ceased.

11-Dehydrocorticosterone implanted as a pellet produced effects qualitatively similar but quantitatively much less than those of 11-dehydro-17-hydroxycorticosterone acetate.

implanted as pellets consisting of one part of œstrogen and three parts of cholesterol. After 30 days the mice were autopsied. The addition of cholesterol to the œstrogens decreased the rate of absorption about 300- to 400-fold. The œstrogens increased the body weight at the lower dose but inhibited or decreased it at the higher dose. The kidneys were not or only slightly increased in size. The thymus was decreased and the seminal vesicles and prostate were increased about the same by both doses of œstrogens. The arginase activity of the kidney was increased equally by both dose levels. The arginase activity of the liver was not remarkably increased.

EFFECT OF ŒSTROGEN ALONE AND IN COMBINATION WITH TESTOSTERONE ON THE BODY AND ORGAN WEIGHTS AND THE ARGINASE AND PHOSPHATASES OF THE ORGANS OF THE MOUSE. *Amer. J. Physiol.*, **155**, 265-271 (1948) Charles D. Kochakian, E. E. Garber and Mary N. Bartlett.

Male mice castrated at 17–19 grams body weight were implanted subcutaneously with a pellet of testosterone, α-œstradiol, methoxybisdehydrodoisynolic acid (MDDA), 1-methylœstradiol or 1-methylœstrone. The first two œstrogens also were implanted as pellets consisting of one part of œstrogen and three parts of cholesterol, and simultaneously with a pellet of testosterone. All of the experiments were for 16 days. In addition, the testosterone and α-œstradiol experiment was performed at 10 and 30 days. The rate of absorption of MDDA was about eight times, and that of 1-methylœstradiol, four times that of α-œstradiol. The introduction of the 1-methyl group into œstrone did not alter its rate of absorption from a pellet. The simultaneous implantation of a pellet of testosterone did not influence the rate of absorption of α-œstradiol or MDDA.

MDDA and α-œstradiol, as pure pellets, greatly reduced the body weight in a manner resembling inanition. There was a concomitant retention of urine which was exacerbated by the simultaneous administration of testosterone. Also deaths occurred only in those mice that were implanted

or a "standard" prepared diet to 24-hour fasted adult male rats for 10 hours caused a deposition of liver glycogen and the expected changes in urinary nitrogen and urea excretion, but did not change the activities of the arginase, "alkaline" (*pH* 9·8) or "acid" (*pH* 5·4) phosphatases of the liver or kidney. The feeding of 30 per cent glucose by stomach tube at one- or two-hour intervals also increased the liver glycogen without any changes in the activities of the liver or kidney enzymes.

The feeding of the above diets for seven days at 10 mg./day *to normal 250-gram male rats showed that the high carbo*hydrate diet caused a loss in body and kidney weight but no change in enzyme activities. The livers of these animals lost weight and protein, but contained a large amount of glycogen. There was a decrease in arginase but a moderate increase in "alkaline" phosphatase. The high protein diet, on the other hand, maintained the body weight and increased the kidney weight and protein, with a concomitant increase in the enzymes. The liver weight, protein and enzymes were somewhat increased. The glycogen content, however, was only one-half that present in the livers of the rats fed the high carbohydrate diet.

The feeding of the high protein diets as above to completely and partially adrenalectomized, castrated rats increased the kidney and liver weights but did not change enzyme activities of these organs. Organ and enzyme changes are not comparable to those after administration of protein anabolic or catabolic steroid hormones

Œstrogens

EFFECT OF ŒSTROGENS ON THE BODY AND ORGAN WEIGHTS AND THE ARGINASE AND "ALKALINE" AND "ACID" PHOSPHATASES OF THE LIVER AND KIDNEY OF CASTRATED MALE MICE. *Amer. J. Physiol.*, **151**, 126-129 *(1947) Charles D. Kochakian*

Male mice castrated at 16·0 to 19·5 grams body weight were implanted subcutaneously with a pellet of pure œstrone, equilin, œstriol, α-œstradiol, α-œstradiol benzoate and α-œstradiol dipropionate. The first four œstrogens also were

The characteristic protein anabolic and enzymic effects of testosterone propionate were not impaired by the diabetes.

DISCUSSION

Elson: There seems to be some divergence of opinion between this country and the United States as to what constitutes a high protein

content above 20 per cent has very little effect upon succinoxidase;

logical value of the protein you are using; casein definitely does not

needs. We have also used manganese, and have used pre-incubation

with both testosterone and pure pellets of MDDA or α-œstradiol.

The renotrophic property of testosterone was not altered, but the androgenic property was greatly reduced by both α-œstradiol and MDDA. The increase in arginase activity produced by testosterone and MDDA or α-œstradiol summated when the androgen and either of the œstrogens were administered simultaneously. The œstrogens did not influence the effect of the androgen on either "alkaline" or "acid" phosphatase. MDDA at the high dose produced a remarkable increase in the arginase activity of the liver; α-œstradiol was ineffective. MDDA also produced a remarkable increase in the "alkaline" phosphatase at the high dose and α-œstradiol a moderate increase at its high dose. MDDA stimulated a small increase in "acid" phosphatase at the high dose; α-œstradiol was ineffective. 1-Methylœstradiol and 1-methylœstrone were ineffective in all of the above tests.

Diabetes

EFFECT OF TESTOSTERONE PROPIONATE ON TISSUE ENZYMES OF DIABETIC RATS. Unpublished. Charles D. Kochakian, Phyllis M. Wright and Evangeline Robertson

The extra endogenous protein metabolism occurring in phlorrhizin and alloxan diabetes was not sufficient to change significantly the liver arginase or phosphatase activities of normal or castrated adult male rats. Moreover, no correlation between the degree or duration of the diabetes and the enzyme activities of the liver could be detected. Tremendous increases, 400-600 per cent, in protein ingestion, however, stimulated an increase in the arginase activity of the diabetic as well as the non-diabetic rats. Smaller increases, 100 to 200 per cent, produced increases in arginase activity concomitantly with the increase in liver weight.

The kidney weight and nitrogen content was increased but the enzymes were not changed except in those rats that had considerable sclerosis of the tubules after prolonged and severe alloxan diabetes.

KOCHAKIAN: I would expect that. *That fits in with our subsequent* experiments on animals treated with phlorrhizin or alloxan, where we [illegible]

[illegible] a certain [illegible] I haven't [illegible]

KOCHAKIAN: It's broken down to ammonia nitrogen, and in an animal that forms protein, that must go into protein.

FRENCH: But I think it's very doubtful that this is from urea.

FOLLEY: Some work on the ruminant points to the utilization of urea by the protozoa and bacteria of the rumen. The theory is that the bacteria eventually die and their cellular proteins are digested by proteases of the alimentary tract and are absorbed into the body as amino-acids.

KOCHAKIAN: Has anybody studied these protozoa to see how they do it?

FOLLEY: I don't know.

[illegible]

KOCHAKIAN: None that I know of. We did some paper chromatography in experiments on hamsters which had been treated with androgen and found in these pilot experiments nothing remarkable in the blood amino-acids.

YOUNG: They're apt to be deceiving. Of course, the tissue amino-acids would be of greater interest, but it is rather difficult to get experimentally an index of the integration of, say, the liver free amino-acid concentration over the whole of an experiment.

[illegible]

In a recent paper by Dunn in California in which he studied the tissues

with both cobalt *and manganese*. Manganese will give higher absolute values. Pre-incubation at 37° will give further increases and pre-

100 per cent with the cobalt method with an incubation period of 6 *hours at 37°, the same increase, within 10 per cent, would be obtained* by the use of $MnCl_2$ in the *determinations*.

Our main interest in trying out manganese was to see if we could possibly show it to be a different activator, and as yet we haven't. It looks as if it is a parallel phenomenon. If you use both together, so that the molarity would be the same or even greater, you will find that the cobalt will decrease the *effectiveness of the manganese. I think* this needs further exploration.

If you run

hour, then

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rminations

h appeared

in your rats in the war, did you notice it at all in the walls of the blood vessels?

tained on salt. This is a rather longer period than you

TISSUE ARGINASE IN RELATION TO THE ADRENAL CORTEX AND DIABETES

A. L. GREENBAUM

THIS communication is a brief summary of the work that was done in Dr. Folley's laboratory at Shinfield on arginase in relation to lactation and diabetes between 1945-48.

The earliest experiments were a study of the changes in the liver and mammary gland arginase of lactating rats which had been adrenalectomized and maintained on normal and high protein diet (Folley and Greenbaum, 1946). The results were essentially similar on both diets, in that adrenalectomy caused a profound decline in the level of arginase in both liver and mammary gland. Treatment of these animals with adrenal cortical steroids, 17-hydroxy-11-dehydrocorticosterone (Kendall's Compound E) and 11-dehydrocorticosterone (Kendall's Compound A), as well as deoxycorticosterone acetate (DCA), restored the liver arginase level toward normal in all three cases, but failed to raise that of the mammary gland. In this latter tissue it was felt that the failure to achieve any significant restoration could be attributed to the methods available at that time. When these experiments were performed no effort was made to measure the amount of milk in the gland. Milk acts as an inert diluent in the enzyme assay, causing under-estimation. If these measurements could be repeated using the methods now available to correct for this milk error, there is little doubt that the cortical steroids would be active in restoring the arginase toward control levels. None of the cortical steroids effected complete replacement in the liver, but DCA was the most effective of those tried.

The failure to find an increased liver arginase on the high protein diet was rather surprising in view of the results of

of a number of species of animals, it appeared that the amino-acid contents, determined by the bacteriological method, in various tissues were very similar. I am wondering just how far we can get with that [illegible] It seems to me more likely that the amino-acids are [illegible]

[illegible] you [illegible] with [illegible]

growth [illegible] our ex-

Koc[illegible] e, even

perime[illegible] nabolic

though [illegible]

effect. [illegible]

deamination of amino-acids, and it is on this basis that the results can most profitably be discussed. We can reasonably interpret the results after adrenalectomy in terms of decreased protein catabolism. It must be admitted that the superior replacement therapy provided by DCA in the first experiment described is rather anomalous on this explanation, but this can most reasonably be accounted for on the grounds that an adrenalectomized rat with a salt and water balance severely upset is in a semi-moribund state, and functions, such as deamination, must be proceeding at a very low ebb. In such a rat the DCA therapy restores the salt and water balance and thereby immensely improves the general well-being of the animal. Increased deamination is, in this context, not so much a specific effect of DCA, but rather a secondary effect, arising from the improved physical condition of the animal.

These experiments on replacement therapy, together with the increases that one finds in liver arginase of the diabetic rat, that is, in rats in which gluconeogenesis presumably is proceeding in full measure, lead us to the conclusion that the changes in liver arginase level are secondary to the rate of deamination. They provide no support at all for the conception of a direct connection between the adrenal steroids and liver arginase itself. The results of Russell and Wilhelmi (1940) do seem to show such a direct relationship between adrenalectomy and the rate of deamination by kidney slices. I think the most probable explanation of the change in the liver arginase levels, in our own experiments anyway, lies in the rates at which amino-acids are presented for deamination and the amino groups subsequently presented to arginase for detoxication.

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[illegible]

Lightbody and Kleinman (1939), but the results are in keeping with those of Kochakian.

These preliminary experiments were open to criticism on the grounds that, as arginase is an enzyme which might be considerably influenced by gluconeogenesis, variations in the quantity and the quality of the diet had to be considered. This factor is particularly important in adrenalectomized animals, which invariably suffer from anorexia as a result of the operation. Further, the upset of the electrolyte balance could also cause severe secondary effects. These various factors were investigated later (Cowie, Folley, French and Greenbaum, 1947: Folley and Greenbaum, 1948), and the effect of variations in the concentration of sodium in the diet, and the dietary sodium-potassium ratio were also studied. The pair-feeding technique was used to minimize effects arising from dietary disturbances. It was demonstrated that restriction of the food intake of the control rats to that of the operated animals failed to cause any lowering of the liver arginase, and anorexia could therefore be eliminated as a cause of the decrease. It was further shown that DCA was the most effective therapy even in rats maintained on high salt diets.

Some further experiments were also reported on the level of liver arginase in experimental diabetes (Folley and Greenbaum, 1949). Rats were made diabetic with alloxan, left for 10 days in metabolism cages, and then killed. Over this period they excreted about 1 86 g. of sugar a day, and this was accompanied by a considerable increase in the urinary urea, suggesting that the sugar excreted was derived from protein by gluconeogenesis. Measurement of the liver arginase of these rats revealed that it had increased considerably over that of the control rats.

The results of the two sets of experiments, considered [illegible] the [illegible] it is [illegible] the [illegible] only well authenticated function so far described is that it is involved in the detoxication of ammonia produced by the

FOLLEY: You did get an increase in the arginase content per gram of liver?

GREENBAUM: Yes, we got an increase per gram of liver, but then the liver is decreased in weight markedly.

KOCHAKIAN: If the liver weight had gone down, I'm quite prepared to call that "no change."

GREENBAUM: Yes, I think we can say "no change." That's why I neglected to mention the experiments.

FOLLEY: The kidney alkaline phosphatase went up, which is rather [illegible]

[illegible]

tosterone, but that wasn't successful.

WILLIAMS-ASHMAN: Have you ever done any experiments on localization of arginase? You've no idea whether it is in the mitochondria?

GREENBAUM: We have no idea.

[illegible]

superior.

BUSH: You can't very well explain the advantage of DCA in terms of salt retention activity.

[illegible]

adrenalectomy is one of the most appalling biochemical procedures at best anyway; it's such a drastic procedure It's as though you eviscerated an animal entirely and replaced four inches of colon in the hope of [illegible]

[illegible]

KOCHAKIAN: He just published some *in vivo* work, too

[illegible]

have looked at operated animals and suspected that I have left behind that little bit of capsule that might be left on the blood vessel, looked

DISCUSSION

KOCHAKIAN: What kind of diet did you use in these diabetic animals?

GREENBAUM: Stock diet.

KOCHAKIAN: We have yet to see a definite increase in liver arginase either with our standard diet or with the high protein diet. I don't think your diabetic animals are as diabetic as ours. We've had animals putting out as much as 10 g sugar daily. The only large increase in arginase that I've seen was in an animal with an ovarian tumour.

FOLLEY: We have been trying for some time to get partially depancreatized rats to excrete more sugar, but the strains of rat that we've tried are all peculiarly resistant to the operation and we've never been able to get much sugar excretion.

KOCHAKIAN: How long have you carried them after you've depancreatized them?

FOLLEY: We depancreatized them at, I think, 60 g. and carried them up to—was it 200 g., Dr. Greenbaum?

GREENBAUM: At first we pancreatectomized an older animal, kept it for about 6 weeks. Then we tried pancreatectomizing them at only ... the body ... ally they

... n't know ... boratory?

FOLLEY: Yes. I should say that Dr. Greenbaum hasn't mentioned that we have also done one or two experiments on phlorrhizin diabetes.

GREENBAUM: I think they're not so clear-cut. Phlorrhizin must have a *toxic effect on the livers because they decreased* in size very considerably.

KOCHAKIAN: I think ours showed an increase in size. We used a little benzyl alcohol to make a smooth suspension of it. We have given doses of 30, 60, and 90 mg per day. Sixty milligrams per day gives close to the maximum response.

GREENBAUM: I don't think we're far off that, 50 mg. a day. We've dissolved ours in propylene glycol, which is a very good solvent incidentally.

KOCHAKIAN: Very irritating to the skin.

... They didn't seem to mind unduly and there was no

...

rats?

SUCCINIC DEHYDROGENASE AND ANAEROBIC GLYCOLYSIS IN THE LIVERS OF DIABETIC LACTATING RATS

R. K. MEYER

LACTATING rats were made diabetic by the intravenous injection of alloxan. Rats which were diabetic for five or eight days showed an increase in liver succinic dehydrogenase activity as much as 100 per cent over that of normal lactating rats.

The elevated enzyme activity is correlated with larger numbers of mitochondria in the cells of the livers of the diabetic rats.

A smaller increase (31 per cent) is found in anaerobic glycolysis of diabetic lactating livers when compared with control animals. No increase in either enzyme system was found in the brain tissue of the diabetic rats.

REFERENCE

SHIPLEY, E. G., MEYER, R. K., COPENHAVER, J. H., and MCSHAN, W. H (1950). *Endocrinology*, 46, 334.

DISCUSSION

KOCHAKIAN: How long were these animals diabetic?

off too.

KOCHAKIAN: It looks like an effect upon the pituitary.

MEYER: That's right.

for it at autopsy and couldn't find it, and then did the liver arginase and confirmed my suspicion.

MEYER: What do you find in mice?

KOCHAKIAN: I would suspect that you find the same thing. I haven't done any arginase studies in adrenalectomized mice.

MEYER: There are reports from Bar Harbor that about 50 per cent of some strains of mice have accessory adrenal tissue.

KOCHAKIAN: I would like to see that confirmed by doing the arginase. I think it is possible to have some tissue that looks very much like adrenal tissue but does not have a complete adrenal cortical function.

to alterations in the adenosine triphosphatase activity, which could conceivably limit the overall glycolytic rate?

MEYER: No. I have not. Only this overall method. I might add that Potter and his group have, with some of the carcinogenic dyes that they use in producing hepatomas, also found increased mitochondria.

KOCHAKIAN: There again it's the same problem, is it a direct or an indirect effect of alloxan on the pituitary? Might you be producing a *pseudo-hypophysectomy by alterations in nutrition due to the alloxan* diabetes?

MEYER: In human diabetics their reproductive processes are normal. An experiment we should do is to administer alloxan and clamp off the vein to the pancreas and then do these studies subsequently. That would control the alloxan effect. We have just given a single intravenous injection of 75 mg per kg., and according to the literature the effects *are over in a few minutes. We haven't done that type of experiment* because the circumstantial evidence would indicate that the effect on the pituitary gonadotrophin and the gonads and accessories is due to the diabetes. We've done complete autopsies on these rats, and the adrenal glands are in most animals markedly hypertrophied by eight to ten days. So if there is an interference with pituitary gland secretion it must be differential.

ELSON. I take it that the *succinic dehydrogenase is the* dehydrogenase component of the succinoxidase enzyme system that you estimate by the methylene blue technique?

MEYER: It was measured by the Potter method with cytochrome *c*, aluminium chloride and substrate added.

ELSON. Have you tried just using the methylene blue instead of cytochrome c?

MEYER. No.

ELSON Because if it is mainly dehydrogenase component, we have shown that that component is very sensitive to the amount of protein in the diet, in fact, the amount may to some extent measure the amount of protein synthesis going no. I am wondering if you might be getting a reversal of the sort of thing I suggested with the growth inhibitors. Perhaps the availability of something like energy-rich phosphate is affected, and is not being used for increased carbohydrate metabolism but for extra protein synthesis, which shows up in the increase in the succinic dehydrogenase.

WILLIAMS-ASHMAN. *Have you measured the catalytic activity of* your mitochondria per mg of nitrogen? Is that increased over the mitochondria isolated from normal tissue? Or are you sure it's just more mitochondria per unit of the homogenate?

MEYER We have determined it on a nitrogen basis, but the volume on centrifugation measured in the modified *hæmatocrit tube and the* histological studies show a greater volume of mitochondria, or large granules.

WILLIAMS-ASHMAN I am very interested in that, because of course one way of elevating the liver succinoxidase activity is to administer thyroxine, and some preliminary work by Dr J. D Judah suggests that in that case the total amount of mitochondria is not greatly altered, but the *mitochondria themselves* seem to be more catalytically active in terms of either nucleic acid phosphorus or milligrams of nitrogen. *You measured* your anaerobic glycolysis in homogenates with hexose diphosphate as substrate. Do you think your increase could be relate-

is being expended in this field, particularly in regard to arginase, the best-known of the phosphomonoesterases, and also some of the respiratory enzymes like the succinic-oxidase system. That type of research has been described in many of the papers presented at this meeting, and we can feel sure, from what we've heard, that considerable progress is being made along these lines.

I feel that it must be regarded as significant that the most fundamental field of all, namely the study of the effects of steroid hormones and other hormones such as the trophic hormones of the anterior pituitary on isolated enzyme systems has not been touched on at all at this conference That is for the very good reason that we know practically nothing about the way in which hormones act in the living body. A start has been made with the studies of Cori and his followers on the mechanism of the action of insulin, and we may expect that greater progress will soon be made in this field, which I personally consider the most fundamental field remaining in endocrinology, namely the elucidation of the intimate mechanism of hormone action. All we can say at present, from what has been said at this meeting and from what we know, is that hormones probably act by modifying the action of enzyme systems.

We may hope that this conference, which for all I know may be the first of its kind on this subject, certainly the first held in this country, will stimulate further research and encourage others to take up this topic, so that we may expect rapid progress towards solving the fundamental problem of how hormones actually govern metabolic processes within the living cell.

CHAIRMAN'S CLOSING REMARKS

S. J. FOLLEY

In commenting on the proceedings of this conference, which has dealt with the interactions of hormones and enzymes, a subject of ever-increasing interest, I would point out that three main fields of endeavour can be distinguished.

First of all, there is the study of enzymes which are believed to be concerned in the metabolism of hormones, a consideration which in this conference has been limited to one enzyme, glycuronidase, about which we have had a number of communications. Since the œstrogens and the progesterone derivative, pregnanediol, seem to be excreted, at any rate partially, in the form of conjugates with glycuronic acid, there might be some reason to think that perhaps β-glucuronidase is concerned with the metabolism of the female sex hormones. However, we have heard contributions which indicate that this enzyme probably has a wider implication in metabolism than that. For instance, it may be concerned with cellular growth, and the opinion has been expressed that glycuronidase may not be responsible for the synthesis of the glycuronides of the sex hormones. It seems rather unfashionable nowadays to postulate a simple synthetic role for hydrolytic enzymes within the body. Thus at one time we thought that alkaline phosphatase might be concerned in phosphorylation by simple reversal of its hydrolytic action, but this is no longer a fashionable point of view. Nevertheless Dr. Kochakian doesn't seem to be afraid to postulate it in connection with the action of arginase.

The second field, represented again by a number of papers, is the study of the changes in the enzyme equipment of various tissues in relation to the metabolic effects of steroids, and in particular the sex hormones. At the present time much effort

is being expended in this field, particularly in regard to arginase, the best-known of the phosphomonœsterases, and also some of the respiratory enzymes like the succinic-oxidase system. That type of research has been described in many of the papers presented at this meeting, and we can feel sure, from what we've heard, that considerable progress is being made along these lines.

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